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Hemoglobins of the Cutthroat Trout Salmo clarki

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HEMOGLOBINS OF THE CUTTHROAT TROUT

SALMO CLARKI

by

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Jonathan N. Southard

TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

Figure Page

Figure Page

ABBREVIATIONS USED

ADP	Adenosine-5'-diphosphate
AMP	Adenosine-5'-monophosphate
ATP	Adenosine-5'-triphosphate
Bis-tris	Bis-(2-hydroxyethyl) imino-tris-(hydroxymethyl)methane
CM	Carboxymethyl
DEAE	Diethylaminoethyl
DPG	2,3-Diphosphoglyceric acid
EDTA	Ethylenediaminetetraacetic acid
Gly	Glycine
GDP	Guanosine-5'-diphosphate
GMP	Guanosine-5'-monophosphate
GTP	Guanosine-5'-triphosphate
Hb	Hemoglobin
IEF	Isoelectric focusing
pI	Isoelectric point
PEI	Poly(ethylene)imine
Taps	N-Tris-(hydroxymethyl) methyl-3-aminopropane sulfonic acid
TCA	Trichloroacetic acid
Temed	N,N,N',N'-Tetramethylethylenediamine
Tes	N-Tris-(hydroxymethyl) methyl-2-aminoethane sulfonic acid
Tris	Tris-(hydroxymethyl) aminomethane

ABSTRACT

Hemoglobins of the Cutthroat Trout

Salmo clarki

by

Jonathan N. Southard, Doctor of Philosophy

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Major Professor: Dr. Thomas M. Farley

Department: Chemistry and Biochemistry

Nine hemoglobins have been isolated from the blood of cutthroat trout. All nine hemoglobins bind oxygen cooperatively and appear to be tetramers with molecular weights of ~64,000. The oxygen equilibria and subunit structures of the purified hemoglobins were studied. In addition, the red blood cells of cutthroat trout were examined for the presence of ATP and GTP, which are known to be physiological modulators of hemoglobins in fishes.

Five hemoglobins with isoelectric points from 9.1 to 7.0 are classified as cathodal hemoglobins. These five hemoglobins have identical oxygen binding properties by the criteria tested. All have oxygen equilibria which are unaffected by protons and ATP and essentially independent of temperature, with overall enthalpies of oxygenation ~0. Two hemoglobins with isoelectric points near 6.5, classified as anodal hemoglobins, have oxygen binding properties distinctly different from those of the cathodal hemoglobins. Both

are characterized by a Root effect, displaying non-cooperative oxygen binding and low oxygen affinity at pH 6.2. ATP causes a large reduction in the oxygen affinity without affecting the cooperativity of oxygen binding. GTP has a similar but slightly larger effect on both hemoglobins. The oxygen equilibria of the anodal hemoglobins are temperature dependent, with the oxygen affinity being reduced as temperature increases. The overall enthalpy of oxygenation is -14 kcal/mol for both hemoglobins. The two remaining hemoglobins represent only a small percentage of the total hemoglobin. These hemoglobins are tentatively designated as embryonic hemoglobins based primarily on a comparison of their properties to those observed for hemoglobins from newly-hatched rainbow trout (Iuchi, I. (1973) Comp. Biochem. Physiol. **44B**, 1087-1101). These two hemoglobins have isoelectric points near 5.9 and oxygen binding properties similar to those of the cathodal hemoglobins.

With the possible exception of one of the embryonic hemoglobins (for which globins were not obtained), all the hemoglobins are composed of two different types of globin chains. Six are $\alpha_2\beta_2$ tetramers, while two of the cathodal hemoglobins are hybrid tetramers of the type $\alpha\alpha'\beta_2$ and $\alpha\alpha'\beta^s$.

Red blood cells of cutthroat trout contain both ATP and GTP, suggesting that, in contrast to rainbow trout, both nucleotides may be important physiological modulators of hemoglobin oxygen affinity in this fish.

(184 pages)

INTRODUCTION

Hemoglobin is the primary oxygen transport protein in vertebrates and constitutes more than 90% of the protein in the circulating red blood cells or erythrocytes of these animals. Hemoglobin binds oxygen at the respiratory surfaces in the lungs or gills and releases oxygen at the tissues where it is required for cellular metabolism. In addition to oxygen, hemoglobin binds other small molecules and ions. These ligands bind at sites other than those responsible for oxygen binding and are termed heterotropic effectors. Binding of these effectors may significantly alter the equilibrium between deoxyhemoglobin and oxyhemoglobin. The high efficiency of oxygen transport by hemoglobin is due to the interactions between the multiple binding sites on the protein for the various ligands.

The blood of most fishes contains several structurally distinct hemoglobin molecules. In many cases, these multiple hemoglobins have similar or identical oxygen binding properties. In some fishes however, most notably the salmonids (salmons and trouts), two different types of hemoglobins occur. The first type is characterized by anodal mobilities on electrophoresis at pH 8-9. The oxygen equilibria of these anodal hemoglobins are greatly altered by heterotropic effectors and are strongly temperature dependent. The second type of hemoglobin migrates cathodally during electrophoresis at pH 8-9. These cathodal hemoglobins have oxygen equilibria which are less sensitive to heterotopic effectors and less temperature

dependent than for the anodal hemoglobins. These two types of hemoglobins with different oxygen binding properties are thought to serve different oxygen transport functions in the fishes in which they are found. Rainbow trout (Salmo gairdneri) and cutthroat trout (Salmo clarki) have both anodal and cathodal hemoglobins. The two major hemoglobins of the rainbow trout (one anodal and one cathodal) have been extensively studied.

For several years the Utah Cooperative Fishery Research Unit has been conducting biochemical and genetic studies of rainbow trout strains. The overall goal of this research is to investigate adaptive mechanisms which might improve the performance of trout strains either under hatchery conditions or in the wild. In the course of these studies it was observed that cutthroat trout have hemoglobins which are electrophoretically identical to those of rainbow trout except for the presence of three additional cathodal hemoglobins. A question arose as to whether these additional hemoglobins represent an adaptation in cutthroat trout which might be related to the generally superior performance of this fish in the wild as compared to rainbow trout.

The goal of this study was to isolate the multiple hemoglobins of cutthroat trout and measure the oxygen equilibrium of each hemoglobin under a variety of experimental conditions. The primary interest was to determine whether the cathodal hemoglobins of cutthroat trout have oxygen binding properties different than those of the cathodal hemoglobins of rainbow trout. If so, the presence of the additional cathodal hemoglobins might confer a physiological advantage to cutthroat trout, and this characteristic may be useful

as a marker for desirable strains in selective breeding programs.

In addition to studying the oxygen binding properties of the isolated hemoglobins from cutthroat trout, the subunit structures of these hemoglobins were investigated as a first step in determining the genetic relationships between the hemoglobins. Also, the red blood cells of rainbow and cutthroat trout were examined for the presence of nucleotides, specifically adenosine-5'-triphosphate (ATP) and guanosine-5'-triphosphate (GTP), which are physiologically important heterotropic effectors of fish hemoglobins.

LITERATURE REVIEW

General aspects of hemoglobin structure and function—The hemoglobin molecule represents perhaps the most extensively studied and best understood of all the proteins. The great attention that it has recieved is due not only to its physiological importance, but also, due to its polymeric nature and ability to interact with various ligands at different sites, it has been employed as a model for study of protein-ligand interactions in general. Since most ligands which interact with hemoglobin are bound reversibly, these protein-ligand, or allosteric, interactions can be studied under equilibrium conditions, which is frequently not possible with allosteric enzymes themselves. This section provides an overview of the structure and function of hemoglobin as an introduction to the literature of the hemoglobins of fishes which follows. In this discussion, hemoglobin refers to normal human adult hemoglobin (Hb A) unless stated otherwise. For more extensive reviews of structural and functional aspects of human hemoglobin, the reader is referred to Baldwin (1) and Imai (2).

Human hemoglobin is a tetramer with a molecular weight of 64,500, consisting of two α and two β polypeptide chains. In the native protein, each subunit contains a ferrous iron porphyrin (heme) prosthetic group. The three dimensional structure has been determined by high resolution x-ray diffraction analysis (3, 4). The tetramer is tetrahedral in shape and is stabilized by weak interactions, primarily non-polar in nature, between the subunits. The

tetramer can be viewed as two identical $\alpha\beta$ dimers, and the attractive forces within each dimer are stronger than those between the two. The arrangement of the four subunits changes upon binding of oxygen to deoxyhemoglobin. The contacts within each dimer are essentially unchanged, but the contacts between dimers are greatly altered, with the interactions between dimers being much weaker in oxyhemoglobin than in deoxyhemoglobin. There is no contact between the two β chains in either form of the protein. The cavity or crevice between the β chains is larger in deoxyhemoglobin than in oxyhemoglobin.

Hemoglobin binds oxygen reversibly at the four heme groups. A plot of fractional saturation with oxygen (Y) versus the partial pressure of oxygen is typically sigmoidal in shape, indicating that the affinity of the protein for oxygen increases as more ligand is bound, i.e., binding is cooperative. An oxygen equilibrium curve (for cutthroat trout hemoglobin) is illustrated in Fig. 1. This phenomenon of cooperative oxygen binding is termed heme-heme or homotropic interaction, and is more apparent when oxygen equilibrium data is analyzed by means of the Hill plot, log(Y/1-Y) versus log pO_2 (Y/1-Y is the ratio of binding sites with ligand bound to free binding sites). A Hill plot of the oxygen equilibrium curve in Fig. 1 is shown in Fig. 2.

The Hill coefficient, $n_{1/2}$, is defined as the slope of the curve at half-saturation (log (Y/1-Y) = 0), and is related to the degree of cooperativity. If the binding of oxygen to each heme were independent of the other hemes, the Hill plot would be a straight

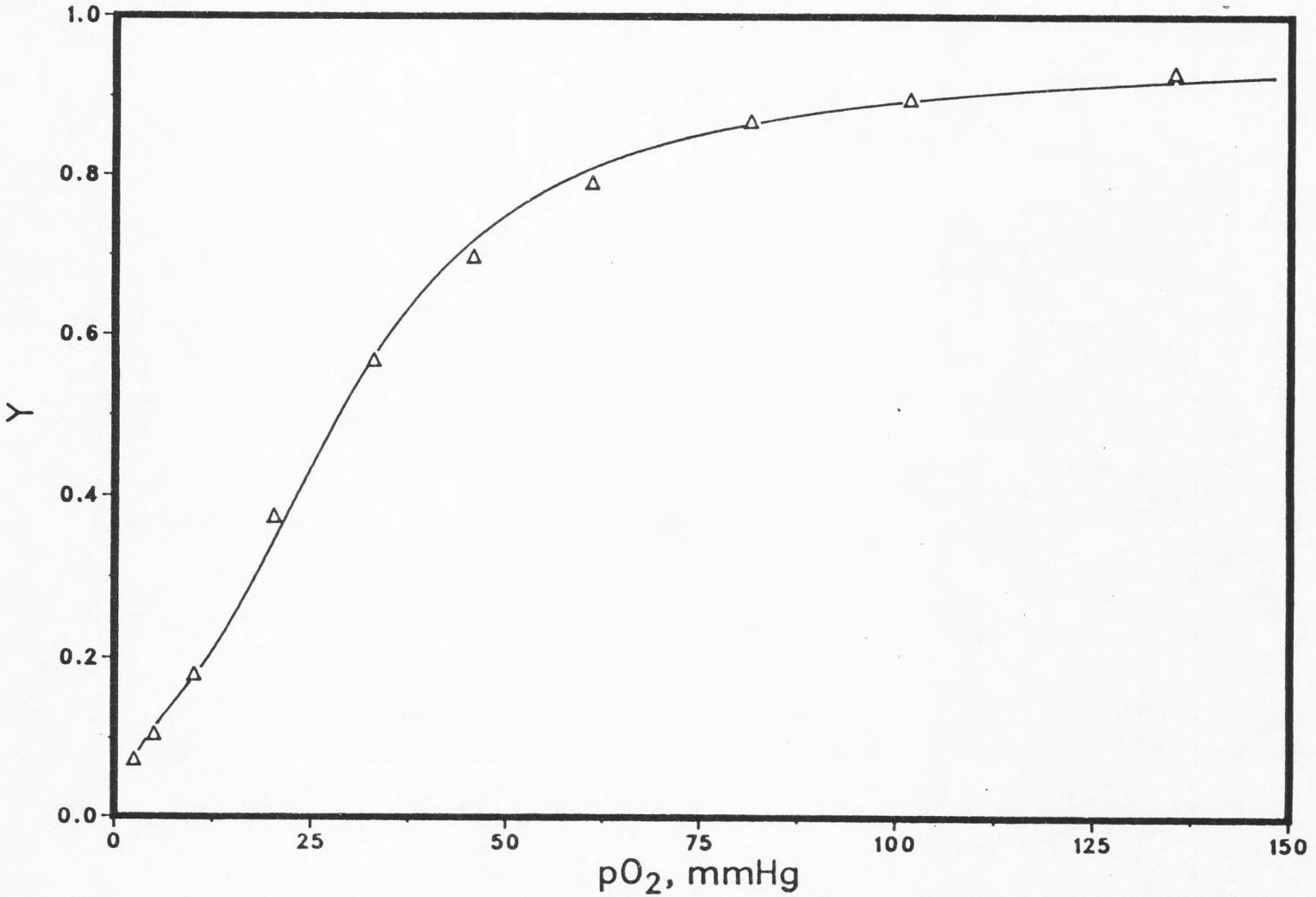


FIG. 1. **Oxygen equilibrium curve of hemoglobin.** The oxygen equilibrium curve for the crude hemolyzate of cutthroat trout at pH 7.1 and 20°C, determined as described in Materials and Methods.

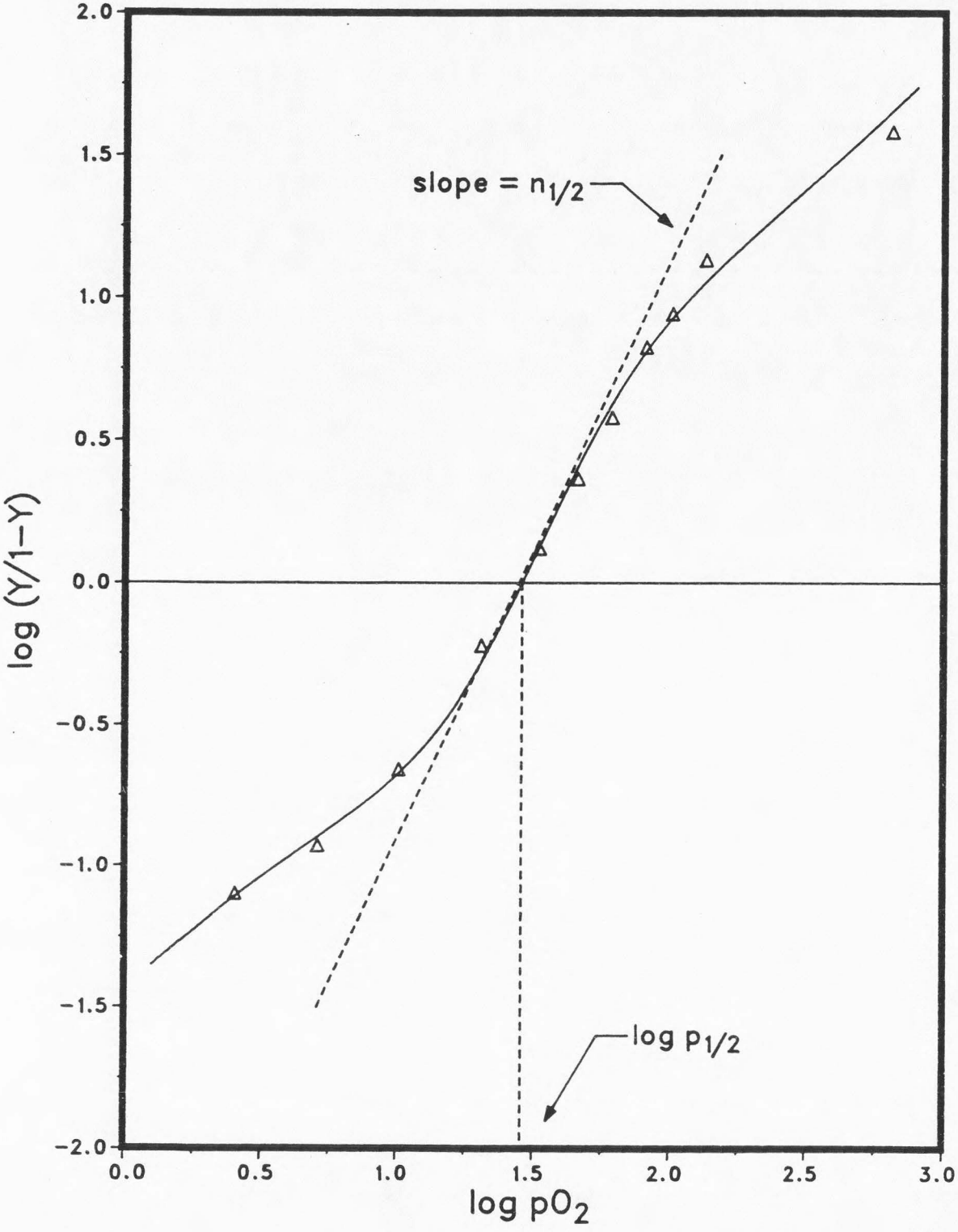


FIG. 2. **The Hill plot for oxygen equilibrium data.** Hill plot of the data shown in Fig. 1, indicating how $n_{1/2}$ and log $p_{1/2}$ are obtained from the curve.

line with a slope of 1. A Hill coefficient > 1 reflects positive cooperativity. Conversely, a Hill coefficient < 1 reflects negative cooperativity, a decrease in affinity as ligand is bound. The position of the curve along the log pO_2 axis reflects the affinity of hemoglobin for oxygen, with the curve being shifted to the left as affinity increases. The value of log pO_2 at half-saturation, log $p_{1/2}$, is used as a measure of the overall affinity for oxygen. Thus the values of $n_{1/2}$ and log $p_{1/2}$ obtained from the Hill plot reflect the cooperativity and affinity, respectively, of oxygen binding to hemoglobin.

The oxygen equilibrium curve of hemoglobin can also be described as the sequential binding of oxygen to the four heme groups of the tetramer, with a separate binding constant for each step:

$$[1] \quad Hb + 4O_2 \overset{K_1}{\rightleftharpoons} HbO_2 + 3O_2 \overset{K_2}{\rightleftharpoons} Hb(O_2)_2 + 2O_2 \overset{K_3}{\rightleftharpoons} Hb(O_2)_3 + O_2 \overset{K_4}{\rightleftharpoons} Hb(O_2)_4$$

According to this scheme, the fractional saturation with oxygen, Y, is given by:

$$[2] \quad Y = \frac{[HbO_2] + 2[Hb(O_2)_2] + 3[Hb(O_2)_3] + 4[Hb(O_2)_4]}{4([Hb] + [HbO_2] + [Hb(O_2)_2] + [Hb(O_2)_3] + [Hb(O_2)_4])}$$

or in terms of the binding constants:

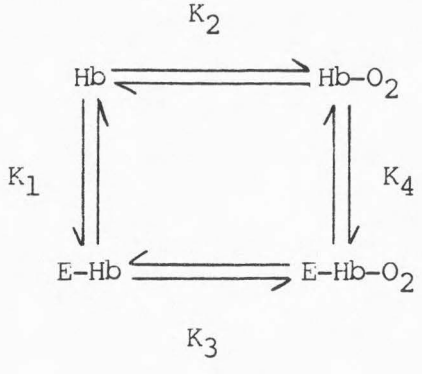
$$[3] \quad Y = \frac{K_1p + 2K_1K_2p^2 + 3K_1K_2K_3p^3 + 4K_1K_2K_3K_4p^4}{4(1 + K_1p + K_1K_2p^2 + K_1K_2K_3p^3 + K_1K_2K_3K_4p^4)}$$

where p is the partial pressure of oxygen. Equation [3] is the Adair

equation (5). The four binding constants can be obtained from the oxygen equilibrium curve. However, this analysis is highly dependent on accurate measurements at both extremes of the curve, which in practice are difficult to obtain (2).

The oxygen equilibrium of hemoglobin is affected by the presence of ions and molecules which display differential binding to oxy- and deoxyhemoglobin. These ions and molecules bind at sites other than the hemes and are called heterotropic effectors. In practice, the major heterotropic effectors of hemoglobin all have a higher affinity for the deoxygenated form of the protein and cause a decrease in the oxygen affinity of hemoglobin.

The thermodynamic basis for the decrease in oxygen affinity in the presence of these heterotropic effectors is illustrated in the simplified equilibrium scheme:



If the affinity of the effector (E) for deoxyhemoglobin is greater than for oxyhemoglobin ($K_1 > K_4$), then the oxygen affinity of deoxyhemoglobin with bound effector must be less than that of deoxyhemoglobin in the absence of effector ($K_3 < K_2$), since $K_1K_3 = K_2K_4$. Thus, in the presence of heterotropic effectors which have a greater affinity for deoxyhemoglobin than for oxyhemoglobin, the affinity of

hemoglobin for oxygen must be decreased. The theoretical aspects of this linkage between the binding of oxygen and heterotropic effectors to hemoglobin have been extensively developed by Wyman (6) and others.

The oxygen affinity of human hemoglobin is pH dependent. As the pH is lowered from pH 9 to pH 7, the value of log $p_{1/2}$ increases from about 0.2 to about 1.2. This (alkaline) Bohr effect is due to a difference in the pK values of several amino acid residues in oxy- and deoxyhemoglobin, with the pKs being higher in deoxyhemoglobin. The amino acid residues involved have been identified as Val 1α, His 122α, His 146β, and Lys 82β(7, 8). As the pH is further lowered below pH 7, the oxygen affinity increases. This acid Bohr effect is less well understood than the alkaline Bohr effect.

Carbon dioxide binds covalently to hemoglobin by formation of carbamates with the N-terminal amino groups of both the α and β chains (9). The β chains have a higher affinity for CO_2 and the binding constant for CO_2 is larger in deoxy- than in oxyhemoglobin (10). In addition to this direct binding, carbon dioxide affects the oxygen equilibrium of hemoglobin indirectly via hydration to carbonic acid:

[4] $$CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons HCO_3^- + H^+$$

The decrease in pH due to the dissociation of carbonic acid causes a decrease in oxygen affinity.

The third major type of heterotropic effector of hemoglobin are various anions, the most important of which are organic phosphates. The large decrease in the oxygen affinity of human hemoglobin in the

presence of 2,3-diphosphoglycerate (DPG) was first demonstrated by Benesch and Benesch (11) and Chanutin and Curnish (12) in 1967. DPG binds to both oxy- and deoxyhemoglobin with a stoichiometry of one mole per mole tetramer, with deoxyhemoglobin having a larger binding constant than oxyhemoglobin (13). In deoxyhemoglobin, DPG binds in the crevice between the β chains, with formation of salt bridges between the negatively charged phosphates and residues Val 1, His 2, Lys 82, and His 143 of both β chains (14). The binding site in oxyhemoglobin is not known. Inorganic anions, e.g., chloride and phosphate, also cause a decrease in oxygen affinity due to preferential binding to deoxyhemoglobin. However, the binding constants for these anions are 100-1000 times smaller than those for the organic phosphates and their effects on the oxygen equilibria are much smaller (2).

The binding of each of the heterotropic effectors discussed above (protons, carbon dioxide, and organic phosphates) is linked not only to the binding of oxygen, but to the binding of the other effectors. The binding site for DPG contains two of the Bohr effect residues (Lys 82 of both β chains) and the two residues (Val 1 of both β chains) which are most important in the binding of carbon dioxide. Thus, these heterotropic effectors interact in a complex manner. As stated by Imai (2, p. 47):

> The combined effects of the non-heme ligands are rather complicated... Those effects are interrelated and none of them can be isolated as a single effect exerted by one ligand. This means that the intact function of hemoglobin is realized as a consequence of integrated effects of all the non-heme heterotropic ligands interacting with hemoglobin in the red cell.

On a physiological level, the heterotropic effectors have an important aspect in common: they all serve to decrease the oxygen affinity of hemoglobin to suit physiological requirements. This is also true for the homotropic interactions between the subunits themselves, as the isolated subunits have much higher oxygen affinities than the intact tetramer (15). In the case of the organic phosphates, the homotropic interactions as well as the overall affinity of hemoglobin for oxygen are altered (16).

The primary function of hemoglobin is to transport oxygen from the lungs to the tissues and carbon dioxide in the reverse direction. In the tissues, the blood pH is lowered due to the hydration of CO_2 produced by cellular metabolism and dissociation of the carbonic acid formed (eq. [4]). The oxygen affinity of hemoglobin is reduced and O_2 is released, accompanied by binding of protons to the resulting deoxyhemoglobin. This uptake of protons shifts the equilibrium in equation [4] to the right, allowing more CO_2 to be dissolved in the blood. At the lungs, the oxygen pressure is high, and hemoglobin binds O_2, with a release of protons. This release of protons shifts the equilibrium in equation [4] to the left, resulting in increased expiration of CO_2. In an analagous manner, in the tissues the carbon dioxide pressure is high and hemoglobin binds CO_2. This reduces the oxygen affinity, and oxygen is released. The CO_2 is released in the lungs when oxygen is bound. This direct transport of CO_2 by hemoglobin is limited by the concentration of DPG in the red blood cell, since CO_2 and DPG compete for the same binding site (the N-terminal amino groups of the β

chains). Direct binding of CO_2 is thought to account for only 10% of the total CO_2 transport (17). DPG is thought to function to adjust the oxygen affinity of hemoglobin to compensate for variations in the supply of oxygen or in metabolic demands for oxygen. Increased levels of red blood cell DPG have been correlated with conditions (anemia, pulmonary disorders, rapid increase in elevation) which lead to a decreased oxygen supply (18).

Other factors in addition to the binding of non-heme ligands affect the oxygen equilibrium of hemoglobin. The effect of temperature, although not physiologically important in warm-blooded animals, may be important in fishes and and other cold-blooded animals subject to thermal variations.

The oxygen affinity of human hemoglobin decreases with increasing temperature, with the value of $p_{1/2}$ increasing by a factor of 1.8 for a 10°C increase in temperature (19). The temperature dependence of the oxygen affinity can be expressed in terms of the overall or average enthalpy (ΔH_{av}) of oxygenation by means of the modified van't Hoff equation:

$$[5] \qquad \Delta H_{av} = 2.303\ R\ \frac{d(\log p_{1/2})}{d(1/T)}$$

where $1/p_{1/2}$ represents the overall (average) equilibrium constant for the binding of oxygen to hemoglobin (6). The calculated value of ΔH_{av} includes not only the enthalpy for oxygen binding, but also the enthalpy for release of any non-heme ligands present, and the heat of solution of oxygen (all values of ΔH_{av} cited are corrected for the heat of solution of oxygen, -3.1 kcal/mol at 20°C, and expressed

in kcal/mol O_2). Thus, ΔH_{av} for human hemoglobin (in the absence of phosphates) ranges from -15.2 kcal/mol at pH 9.1 to -11.1 kcal/mol at pH 6.5 (16), reflecting the greater release of protons (positive ΔH) upon oxygen binding at low pH. The shape of the oxygen equilibrium curve is only slighty affected by changes in temperature (16), indicating that the enthalpy of oxygen binding is essentially independent of the degree of saturation.

Hemoglobins of fishes—The blood of most fishes contains multiple hemoglobins. In an electrophoretic survey of 77 species of teleost (bony or 'true`) fishes (20), 71 of the species examined were found to have multiple hemoglobins with an average of four components for each species. In addition to this multiplicity of hemoglobins, the hemoglobin pattern in ten species was polymorphic, i.e., the number of hemoglobins varied for individual fish from each of these species.

A great deal of research has been directed towards understanding the nature of hemoglobin multiplicity and polymorphism in fishes. An underlying assumption of much of this work has been that the multiple hemoglobins have differing functional properties which allow the fish to survive under various environmental and metabolic stresses. Fluctuations in water temperature, pH, and dissolved oxygen concentrations, and metabolic acidosis and hypoxia induced by bursts of strenuous exercise are thought to be the most important of these stresses. The different hemoglobins may also serve the changing needs of the fish during development from embryo to adult, or in supplying oxygen to specialized organs, e.g., the swimbladder.

Alternatively, the multiple hemoglobins may not by physiologically significant, but may be the result of neutral gene mutations rather than of adaptive selection. In this section, the structural and functional properties of fish (excluding trout) hemoglobins and the possible physiological significance of these properties are reviewed. The hemoglobins of trout are considered separately in the following section.

All fish hemoglobins which have been characterized appear to be tetramers with molecular weights approximately equal to that of human hemoglobin. Several structural features are, however, distinctly different in the hemoglobins of fishes. These structural alterations range from the primary to the quaternary structural level.

Except for one hemoglobin from trout, the complete amino acid sequence of only two fish hemoglobins, the two major hemoglobins of carp, Cyprinus carpio, are known. Both hemoglobins contain two different subunits, designated α and β by analogy to human hemoglobin. The two hemoglobins share the same α chain and differ only in the β subunit.

A comparison of the amino acid sequences of the α chains of carp and human hemoglobins (21) shows that the carp sequence differs in 67 positions and contains two insertions and one deletion for a total of 142 amino acid residues versus 141 for the human α chain. The amino acid sequences of the two carp β chains (22) are very similar, differing in only four positions. Both contain one insertion for a total of 147 amino acid residues compared to 146 for the human β chain. The two carp β chains differ from the human β

chain in 66 and 67 positions.

The N-terminal valine of the human α chain is replaced with acetylserine in the carp α chain (21). This same replacement is found in hemoglobins from Catostomus species (suckers) (23) and the eel, Anguilla rostrata (24). From this and other evidence (25), it appears that the N-terminal residue of the α chains in most, and perhaps all, fish hemoglobins are blocked.

In contrast to human hemoglobin, many fish hemoglobins have been shown to contain more than two different subunits. Tetramers of the type $\alpha\alpha'\beta_2$ and $\alpha_2\beta\beta'$ have been observed in several species (23, 26, 27, 28) and tetramers composed of four different subunits have been reported for several salmonid fish (29, 30).

The interactions between subunits in fish hemoglobins are apparently significantly different than those in human hemoglobin. This is reflected in the tendency of the tetramer to dissociate into dimers. The tetramer-dimer dissociation constant, $K_{4,2}$, for human oxyhemoglobin is approximately 10^{-6} M (31). The subunit interactions are stronger in deoxyhemoglobin, as reflected by the decreased tetramer-dimer dissociation constant ($K_{4,2} = 10^{-12}$ M) (31). Edelstein et al. (32) determined the tetramer-dimer dissociation constants for the hemoglobins of three fishes: Cyprinus carpio (carp), a freshwater teleost; Brevoortia tyrannus (menhaden), a marine teleost; and Prionace glauca (blue shark), an elasmobranch. The tetramer-dimer dissociation constant for oxyhemoglobin from all three species was similar ($K_{4,2} = 10^{-9}$ to 10^{-8} M), and smaller than that for human oxyhemoglobin. The values of $K_{4,2}$ for deoxyhemoglobin

for the three fish were estimated to be 10^{-11} M to 10^{-12} M, approximately equal to $K_{4,2}$ for human deoxyhemoglobin. Thus, the subunit interactions appear to be similar in human and fish deoxyhemoglobin, but the decrease in the strength of these interactions upon oxygen binding is less for the fish hemoglobins than for human hemoglobin.

Fishes with multiple hemoglobins that have been separated and functionally characterized can be grouped into two classes (25, 33) based on the functional properties of the hemoglobins. In class I fishes, the multiple hemoglobins are all functionally similar, having oxygen equilibria which are both pH and temperature dependent. These hemoglobins are designated as 'slow` (based on elution from DEAE-cellulose at pH 8-9) or 'anodal` (based on electrophoretic mobility at pH 8-9) hemoglobins. Class II fishes contain one or more of these slow or anodal hemoglobins and one or more hemoglobins which display oxygen equilibria which are less dependent on pH and temperature. These hemoglobins are designated as 'fast` or 'cathodal` hemoglobins based on the same criteria as for the pH and temperature dependent hemoglobins.

The majority of fishes studied to date belong to Class I (25). Class I fish include carp (34), the Rio Grande cichlid (35), and the plaice, Platessa platessa (36). Fishes which belong to Class II as demomstrated by functional studies of purified hemoglobins are: Catostomus clarkii (23), the eel (24, 37), and three species of Amazonian fishes (38, 39, 40) (and rainbow trout). On the basis of electophoresis of hemoglobins, several species of salmon (29, 30) and trout (41, 42, 43) may also be Class II fishes.

The slow or anodal hemoglobins of many fishes display oxygen

equilibria which are extremely pH dependent, so that the hemoglobins are not completely saturated even in air (pO_2 ~ 150 mmHg) at low pH. This phenomenon, the Root effect, was first described for whole blood of marine fishes in the presence of CO_2 (44). In current usage (25, 45), the Root effect is considered to be an exaggerated Bohr effect, i.e., strictly a pH effect, and is thus distinguished from the specific effects of CO_2 on the oxygen equilibrium of hemoglobin. In addition to the large decrease in oxygen affinity at low pH, the cooperativity of oxygen binding is also pH dependent for Root effect hemoglobins. As the pH decreases, the value of $n_{1/2}$ approaches 1, indicating that oxygen binding is non-cooperative at low pH.

The fact that half of the N-terminal amino groups in fish hemoglobins are probably blocked suggests that the effect of CO_2 on the oxygen equilibria should be smaller than for human hemoglobin, where all four N-termini are available for CO_2 binding (in the absence of phosphates). Although there has been only one report on the effect of CO_2 on anodal hemoglobins of fishes, the results indicate that this is the case. Farmer (46) measured the oxygen equilibria of the anodal hemoglobins from three Amazonian fishes in the absence and presence of CO_2 (pCO_2 = 30 mmHg). All three showed a decreased oxygen affinity in the presence of CO_2 at alkaline pH, with the magnitude of the decrease being approximately one-half of that for human hemoglobin.

The oxygen equilibria of anodal hemoglobins are also affected by organic phosphates. In the nucleated red blood cells of fishes, the most prominent organic phosphate compounds are adenosine-5'-

triphosphate (ATP) and guanosine-5'-triphosphate (GTP) (25,35). Bartlett (47) examined the red blood cell phosphates for ten species of teleost fishes and found that the ATP/GTP ratio ranged from 10 to 0.6. No 2,3-diphosphoglycerate (DPG) could be detected in the red blood cells of these fishes.

In one of the first studies of fish red blood cell phosphates and their effects on hemoglobin, Gillen and Riggs (35) found ATP to be the major organic phosphate in the red blood cells of the Rio Grande cichlid, Cichlasoma cyanoguttatum. ATP was present at approximately the same concentration as hemoglobin (0.76 mol ATP/mol Hb), while DPG was present in much lower concentration (0.05 mol DPG/mol Hb). Addition of either ATP or DPG to the whole hemoglobin (previously freed of phosphates) caused a decrease in the affinity and an increase in the cooperativity of oxygen binding, with the effect of ATP being considerably greater than that of DPG at equal concentrations. Very similar ATP/Hb and DPG/Hb ratios and effects of these phosphates on the oxygen equilibrium were found for another fish, Catostomus clarkii (23).

The effects of ATP and DPG on the oxygen equilibria of the anodal hemoglobins from the European eel, Anguilla anguilla (37) are similar to those reported by Gillen and Riggs. However, the concentration of GTP in the red blood cells is higher than that of ATP, and GTP caused a greater decrease in affinity and increase in cooperativity of oxygen binding than did ATP. This greater effect of GTP on the oxygen equilibrium has also been found to occur with anodal hemoglobins from the American eel, Anguilla rostrata (48), and from several Amazonian fishes (49, 50).

The effects of ATP and GTP on the oxygen equilibria of the anodal hemoglobins of fishes are qualitatively similar to the effect of DPG on the oxygen equilibrium of human hemoglobin. DPG causes changes similar to, but smaller than, those of ATP and GTP in the equilibria of the fish hemoglobins. Conversely, the effect of ATP on the equilibrium of human hemoglobin is similar to, but smaller than that of DPG (51). Based on the similarities of the effects of ATP, GTP, and DPG, and on structural information for fish hemoglobins, Barra, et al. (52) have suggested that the organic phosphate binding site in fish hemoglobins is the same as that in human hemoglobin, ie., the crevice between the β chains.

There is little information in the literature regarding the effect of temperature on the oxygen equilibria of purified fish hemoglobins. Weber and De Wilde (36) purified five anodal hemoglobins from plaice, Platessa platessa. All the hemoglobins displayed a decrease in oxygen affinity with increasing temperature. The enthalpy of oxygenation (in the absence of phosphates) is similar for all five hemoglobins (ΔH_{av} = -14.1 to -11.5 kcal/mol at pH 7.2). A similar temperature dependence (ΔH_{av} = -12.9 kcal/mol) has been reported for a mixture of two anodal hemoglobins from Anguilla anguilla (37). From this limited data, it appears that the anodal hemoglobins from fish have temperature dependences similar to, but somewhat larger in magnitude than that of human hemoglobin. This conclusion is supported by determinations of the enthalpy of oxygenation of the whole hemoglobins of 7 species of fish in which anodal hemoglobins account for most or all of the total hemoglobin

(ΔH_{av} = -12.7 to -10.6 kcal/mol at pH 9.0) (53).

In summary, the anodal or slow hemoglobins of fishes are characterized by oxygen equilibria which are greatly affected by protons and organic phosphates, with the oxygen affinity being decreased with increasing concentrations of these heterotropic effectors. Protons cause a decrease in the cooperativity of oxygen binding, while organic phosphates cause an increase. In contrast, CO_2 has only a small effect on the oxygen equilibria, as predicted from the known structural features of these hemoglobins. Although it appears that the organic phosphates act by binding to the same site as in human hemoglobin, the structural basis of the Root effect appears to be considerably different than that of the Bohr effect in human hemoglobin. The N-terminal valine of the α chain of human hemoglobin, which is responsible for about 25% of the Bohr effect (8), is replaced by acetylserine in at least some of these anodal hemoglobins. Recently, Perutz and Brunori (54) have proposed that the Root effect of fish hemoglobins arises from amino acid substitutions at five positions, all on the β chains. The oxygen equilibria of the anodal hemoglobins are temperature dependent, with enthalpies of oxygenation comparable to that of human hemoglobin.

The oxygen binding properties of cathodal hemoglobins from fishes are significantly different than those of the anodal hemoglobins discussed above. One of the first cathodal hemoglobins to be purified and characterized is one of the two cathodal hemoglobins from the sucker, Catostomus clarkii (23). The oxygen equilibrium of this hemoglobin is independent of pH from pH 7.2 to 6.7. For comparison, the combined anodal hemoglobins from C. clarkii

show a large decrease in oxygen affinity (log $p_{1/2}$ = 0.25 at pH 7.2 and 0.9 at pH 6.7) in this pH range. In the presence of ATP, the oxygen eqiulibrium curve of the cathodal hemoglobin is shifted to the right (lower affinity) while the shape of the curve is unchanged. The decrease in affinity in the presence of ATP is nearly identical to that of the anodal hemoglobins, but for these hemoglobins the shape of the curve is also affected, with $n_{1/2}$ increased in the presence of ATP.

Gillen and Riggs (24) studied the cathodal hemoglobin from the eel, Anguilla rostrata. This hemoglobin has a reverse Bohr effect (increased oxygen affinity at low pH). In the presence of ATP, the oxygen affinity is decreased, cooperativity is increased, and the reverse Bohr effect is abolished. Apparenty, ATP binds to deoxyhemoglobin in a manner which eliminates the differential proton binding responsible for the reverse Bohr effect. Essentially identical results were found for the cathodal hemoglobin from the eel, Anguilla anguilla (37) (it is uncertain whether A. rostrata and A. anguilla are two distinct species or different populations of the same species). In addition, it was shown that the effect of GTP is similar but greater in magnitude than that of ATP for this cathodal hemoglobin.

The oxygen equilibria of the cathodal hemoglobins from three species of Amazonian fishes, two catfish, Hoplosternum littorale (38) and Pterygoplichthys pardalis (39), and a charachid, Mylossoma sp. (species uncertain) (40), are similar to that of the cathodal hemoglobin from the eel in that both show a small reverse Bohr effect in the absence of organic phosphates. With both of these

hemoglobins however, addition of ATP results in a reversal of the pH effect, so that a small decrease in the oxygen affinity occurs at low pH in the presence of ATP. Also in contrast to the eel hemoglobin, ATP affects only the oxygen affinity with the value of $n_{1/2}$ essentially unchanged.

The effect of CO_2 on the oxygen equilibria of the cathodal hemoglobins of the eel (24) and H. littorale (46) have been determined. For both of these hemoglobins, CO_2 causes a small decrease in oxygen affinity, similar to the effect seen for anodal hemoglobins.

With the exception of the cathodal hemoglobin from rainbow trout, the temperature dependence of the oxygen equilibrium of only one cathodal hemoglobin has been studied. The oxygen affinity of the cathodal hemoglobin from the eel (37) decreases as temperature is increased. The magnitude of this effect (ΔH_{av} = -10.2 kcal/mol) is slightly less than for the anodal hemoglobins which have been studied. The decrease in temperature dependence of the oxygen equilibrium is more dramatic for the cathodal hemoglobin from the rainbow trout ($\Delta H_{av} \sim 0$).

From these studies it can be seen that the oxygen binding properties of the cathodal hemoglobins vary somewhat from species to species, but in all cases, these properties are very different from those of the anodal hemoglobins. the most thoroughly studied cathodal hemoglobin, the most cathodal hemoglobin from the rainbow trout (described in the following section), also fits this description. The oxygen equilibrium of this hemoglobin is virtually unaffected by either protons or organic phosphates from pH 6 to pH 9. The decreased sensitivity of the oxygen equilibria of the

cathodal hemoglobins to protons is partially explained by the carboxyl terminal amino acid sequence of the β chains. The carboxyl terminal histidine (His 146β) found in human hemoglobin and in the anodal hemoglobins from fishes is replaced by phenylalanine in the cathodal hemoglobins from C. clarkii (23) and A. rostrata (24) (and also from rainbow trout). His 146β is responsible for about 40% of the alkaline Bohr effect in human hemoglobin (8). From amino acid sequence data (23, 24) and immunological studies (55, 56) it is evident that the anodal and cathodal hemoglobin are structurally distinct and probably have no subunits in common in Class II fishes where both types of hemoglobin are found.

The hemoglobin molecule acts as a link between the internal and external environments of the organism in which it functions. In fishes, both of these environments are subject to variations which affect the function of hemoglobin. The concentrations of dissolved oxygen, the ionic composition, and the temperature of the water surrounding the fish may vary widely. The possible magnitude of these variations are obvious in the case of fishes such as salmon or eels, which spend part of their life cycle in the ocean and part in freshwater streams. The fluctuations in environmental conditions may be less apparent but equally important in the case of other fishes. For hemoglobin, the internal environment is the interior of the red blood cell. Although the red blood cells of humans have been extensively studied, little is known about the biochemistry of red blood cells from lower vertebrates. In contrast to mature human red blood cells, fish red blood cells are nucleated and have a relatively high metabolic rate, although the details of the metabolism are unknown

(57). Information is available on the concentrations of organic phosphates in fish red blood cells, but little is known regarding the concentrations of other ligands of hemoglobin.

Hemoglobin is subject to evolutionary pressures so that its functional properties are modified from species to species to meet particular physiological requirements. In mammals, the oxygen affinity of hemoglobin and the magnitude of the Bohr effect are related to the body weight of the animal (58). The lower oxygen affinity and greater Bohr effect in small mammals allows a more efficient transport of oxygen to and carbon dioxide from the tissues which operate at a higher metabolic rate in small mammals. This same kind of trend is observed in fishes (59). More active fishes such as salmon and trout have hemoglobins with a lower oxygen affinity and greater pH dependence than less active fishes such as carp.

Several attempts have been made to correlate the presence of hemoglobins with different functional characteristics in fishes to physiological requirements of these fishes. A major limitation of these studies is the difficulty in extrapolation of observed functional properties of hemoglobin solutions to the function of hemoglobin in vivo. Hemoglobin solutions normally contain the macromolecule and a relatively small number of other components. Within the fish, hemoglobin functions inside the permeability barrier of the erythrocyte membrane, suspended in the plasma. The blood plasma is a complex mixture of many different molecules, the composition of which is subject to both temporal and spacial fluctuations. Thus these extrapolations inevitably require the use of simplifying

assumptions and the danger of oversimplification is always present. Nonetheless, several useful relationships regarding basic functional properties of fish hemoglobins and the physiology of fishes have been developed and these are described briefly below.

It has been widely suggested that the anodal Root effect hemoglobins are involved in the function of the swimbladder of fishes. Many fishes contain a swimbladder, an extension of the gut which may be filled with gas. In most fishes it is thought that the swimbladder functions mainly in maintaining buoyancy, although in some cases this organ has evolved to serve respiratory and auditory functions (60). In some fishes the swimbladder is associated with a gas gland and a system of capillary arteries and veins, the rete mirabile (61). The rete mirabile acts as a counter-current exchanger and lactic acid produced by the gas gland may cause a drop in blood pH sufficient to cause Root effect hemoglobins to release most of their oxygen even in the presence of high oxygen pressures. The swimbladder in these fish may contain up to 90% O_2, with N_2 and CO_2 making up most of the remaining gas (61).

Two recent surveys however, indicate that Root effect hemoglobins are not involved in oxygen secretion to the swimbladder in most fishes. Farmer et al. (62) found that of 48 Amazonian fishes with Root effect hemoglobins, only 10 have functional swimbladders (swimbladders with a rete mirabile). Similarly, Ingermann and Terwilliger (63) observed a Root effect in whole hemolyzates of 17 marine fishes, only one of which has a functional swimbladder. From this evidence it appears that Root effect hemoglobins are important in filling the swimbladder with oxygen and thus maintaining buoyancy

only in a minority of the fishes in which these hemoglobins occur.

The Root effect hemoglobins may be involved in oxygen secretion to the eye in many teleosts. Many fishes have a choroid rete mirabile located behind the retina of the eye (64). The choroid rete is anatomically very similar to the swimbladder rete mirabile. With the exception of one primitive fish, the bowfin (Amia calva), the occurrence of a choroid rete is limited to teleost fishes, being absent in higher vertebrates. The eyes of fishes with a choroid rete have oxygen pressures up to 800 mmHg, while fishes and other vertebrates lacking a choroid rete have much lower oxygen pressures in their eyes (less than 100 mmHg) (64). Presumably, these high oxygen pressures are important in vision in these fishes. Although there is no experimental evidence to support this presumption, at least some of the teleosts which lack a choroid rete do not rely on vision to obtain food (65).

In the 48 Amazonian fishes with Root effect hemoglobins studied by Farmer et al. (62), all but 9 have a choroid rete. In the 9 fishes lacking a choroid rete the magnitude of the Root effect is small. In addition, 13 fishes which lack Root effect hemoglobins also lack a choroid rete. The 17 marine fishes found by Ingermann and Terwilliger (63) to have Root effect hemoglobins all have a choroid rete, while 3 species without Root effect hemoglobins also lack a choroid rete. These results indicate that the Root effect hemoglobins of fishes are involved in the secretion of oxygen against high oxygen pressures in the eyes of many fishes, and in fishes which lack a choroid rete the pH dependence of the hemoglobin

oxygen affinity is often greatly reduced.

A physiological role for the pH insensitive, cathodal hemoglobins was first proposed by Powers and Edmundson in 1972 based on their studies of the hemoglobins of catostomid fishes (23). These fishes belong to two subgenera, Pantostomus and Catostomus. The two subgenera are sympatric, that is, a member of one subgenus is usually found living with a member of the other subgenus. Pantostomus species generally inhabit faster parts of streams while Catostomus species are found in quieter pools and sluggish waters. Starch gel electrophoresis at pH 8.6 of hemoglobins from 5 Pantostomus and 3 Catostomus species shows that all the Pantostomus species contain one or more cathodal hemoglobins while only anodal hemoglobins are observed for the Catostomus species. From these observations, Powers and Edmundson (23, p. 6693) suggest that the cathodal hemoglobins confer a physiological advantage to more active fish which inhabit faster waters:

> We have concluded that the cathodal hemoglobins add to the morphological and behavioral characteristics which are conducive to fast water survival in Catostomus (subgenus Pantostomus) clarkii.
>
> Fish in fast water habitats generally have hemoglobins with low oxygen affinities and a large Bohr effect. Although a large Bohr effect is beneficial in releasing oxygen at the cellular level, it can suppress oxygen binding at the gills when the blood pH is sufficiently low. ....The increase in lactic acid following violent exercise (e.g. in escape from a predator) can result in the death of a fish. The cathodal hemoglobins, without a Bohr effect, may provide an emergency system for C. (P.) clarkii to continue swimming after violent exertions. For example, the cathodal hemoglobins may counter the tendency towards premature or excessive loss of oxygen before the hemoglobin reaches the muscle cells.

This conclusion is supported by the finding that blood pH is significantly lowered in fish forced to swim in a swimming tunnel (45, 66). Also, cathodal hemoglobins appear to be widely distributed in hyperactive fishes such as salmon (29, 30) and trout (41, 42, ,43). Recently, it has been shown that during vigorous exercise, metabolism in muscle of salmon is largely aerobic, while the goldfish, Carassius auratus, (which lacks cathodal hemoglobins) relies heavily on anaerobic muscle metabolism under the same conditions (67). The cathodal hemoglobins may be partly responsible for the greater capacity of salmon for aerobic metabolism during exercise.

As described above, the major organic phosphates in fish red blood cells are ATP and GTP. These nucleotides cause a decrease in the oxygen affinity of most fish hemoglobins. The physiological importance of this effect was first demonstrated by Wood and Johansen (68) for the eel. They found that blood from eels acclimated to hypoxic water (pO_2 = 15-40 mmHg) had a higher oxygen affinity than blood from eels acclimated to normoxic water (pO_2 = 140 mmHg). This increase in oxygen affinity was accompanied by a decrease in the molar ratio of ATP to hemoglobin in hypoxic (ATP/Hb = 0.84) as compared to normoxic (ATP/Hb = 1.54) eels.

Further investigation (37) showed that eel red blood cells contain more GTP than ATP and the decrease in the concentration of GTP in hypoxic eels was greater than that of ATP. It was also found that the relative proportions of the 5 hemoglobins of the eel are the same in hypoxic and normoxic acclimated eels. Thus when the oxygen supply is decreased, blood oxygen affinity in eels is

increased via a decrease in the levels of red blood cell ATP and GTP, not by any alteration of the hemoglobins *per se*. Similar changes in blood oxygen affinity and red blood cell nucleotide concentrations during hypoxia have been observed in several other fishes (69, 70, 71). The effects of nucleotides and protons on the oxygen equilibrium of hemoglobin are interrelated. In the presence of ATP or GTP the Root effect is shifted to higher pH (33). This is illustrated (for the unfractionated hemoglobins of cutthroat trout) in Fig. 3. Thus the concentration of ATP and/or GTP (and H^+) in the red blood cell will determine the effective magnitude of the Root effect *in vivo*.

Changes in temperature affect both oxygen supply and oxygen demand in fishes. As temperature increases, the solubility of oxygen in water decreases, so that oxygen supply is reduced. At the same time, oxygen demand is increased due to the increase in metabolic rate at elevated temperatures. In rainbow trout, oxygen consumption increases from 19 mg kg^{-1} hr^{-1} at 2°C to 120 mg kg^{-1} hr^{-1} at 18°C (72), while the saturated oxygen content of water decreases from 13.8 to 9.4 mg l^{-1}. The means by which fishes adjust oxygen transport in response to temperature changes are complex and poorly understood. In some fishes, acclimation at high temperature results in an increase in blood oxygen affinity accompanied by a decrease in red blood cell nucleotide concentrations (45, 73), while in other fishes these parameters appear to be independent of temperature (45, 74). The relative proportions of the different hemoglobins in some fishes may change during acclimation to different temperatures (74, 75), but the significance of these chages has not been clearly

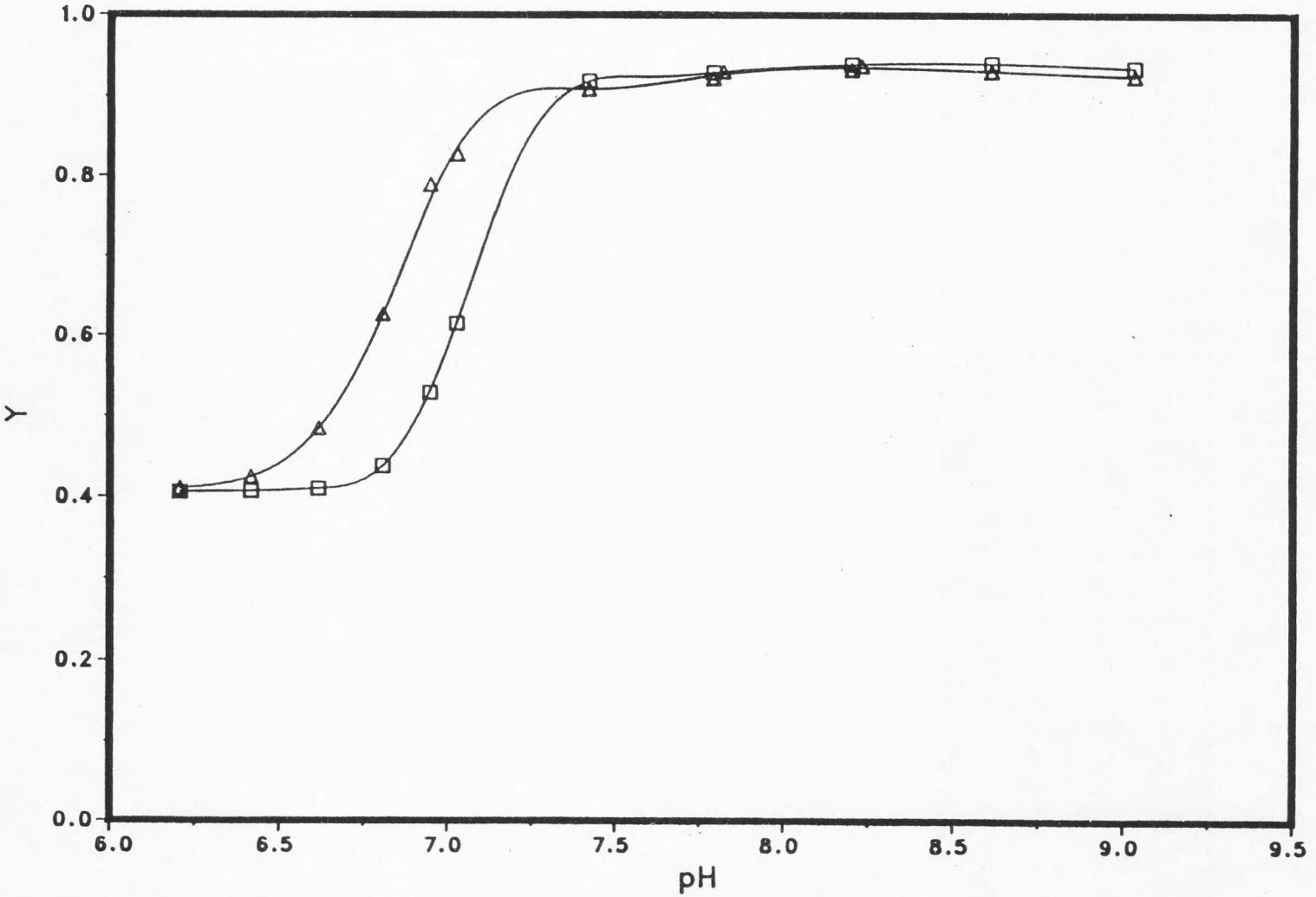


FIG. 3. **The Root effect in the absence and presence of ATP.** Fractional saturation of cutthroat trout whole hemoglobin in air as a function of pH, in the absence (△) and presence (□) of ATP.

demonstrated. Since the oxygen affinities of anodal and cathodal hemoglobins have different temperature and organic phosphate dependences, changes in the relative proportions of these hemoglobins could explain changes in blood oxygen affinity not accompanied by changes in red blood cell nucleotide concentrations (74). This is probably an oversimplification though, as thermoacclimatory responses in fishes appear to involve complex changes in red blood cell metabolism (72), the details of which are not known.

A lack of knowledge of red blood cell metabolism in fishes also limits the understanding of the role of the red blood cell and hemoglobin in the transport of CO_2. In contrast to terrestrial mammals, CO_2 excretion in fishes is regulated primarily to control ionic fluxes (60). At the gills, HCO_3^- and H^+ formed from hydration of CO_2 are excreted in exchange for Cl^- and Na^+. The role of the red blood cell in CO_2 transport is currently subject to debate (60, 76). Carbonic anhydrase, which catalyzes the hydration of CO_2, is present inside the red blood cell but absent from the plasma. In contrast to mammalian red blood cells, the erythrocyte membrane in fishes may be impermeable to HCO_3^- (77) so that intracellular concentrations of CO_2 and HCO_3^- may be very different than those in the plasma. Randall et al. (60) have suggested that the different mechanisms for CO_2 excretion in fishes as opposed to mammals provide a strong selective pressure against the presence of hemoglobins which bind CO_2. This is consistent with the findings that the N-termini of the chains of fish hemoglobins are acetylated and that fish hemoglobins appear to bind CO_2 to a lesser extent than does human hemoglobin (46).

Some of the multiple hemoglobins of fishes may be important during development of the fish from embryo to adult. Differences in electrophoretic patterns of hemoglobins from adult and juvenile fishes have been reported for two species of salmon (29, 30). In both cases, juvenile fishes lack one or more hemoglobins present in adult specimens. Ontogenic variations in hemoglobin have also been reported for the eel (78). In this fish, juvenile specimens contain hemoglobins not present in adults. In the case of both salmon and eel, these apparent changes in hemoglobins may be related to the transition from freshwater to saltwater for young fish of these species. No functional studies have been reported to corroborate speculations regarding the significance of changes in hemoglobin electrophoretic patterns during development in these fishes.

As described above, functionally distinct hemoglobins of fishes may serve several physiological requirements. The extreme pH dependence or Root effect observed for many anodal hemoglobins may be important in delivery of oxygen against high oxygen pressures to the eye of many fishes and to the swimbladder in some others. In addition to these specialized functions, the pH dependence of anodal hemoglobins is of course important in the modulation of hemoglobin oxygen affinity to increase binding of oxygen at the gills and release at the tissues. The greater pH dependence of fish hemoglobins as compared to human hemoglobin may reflect differences in environmental oxygen supplies, metabolic oxygen demands, or red blood cell metabolism in fishes and humans. The cathodal hemoglobins, for which pH has little effect on oxygen affinity, may

serve as an emergency source of oxygen under various conditions, notably vigorous exercise, when red blood cell pH may be to low for the anodal hemoglobins to bind oxygen at the gills. ATP and GTP are important physiological modulators of hemoglobin oxygen affinity in fishes. The different sensitivities of multiple hemoglobins to these nucleotides may be important in the adjustment of blood oxygen affinity in response to fluctuations in environmental conditions (oxygen supply, temperature, etc.).

Many fishes contain multiple hemoglobins which appear to be functionally identical in terms of their oxygen binding properties. Although functional differences may exist which have not been detected, Riggs (25, 79) has pointed out that functionally identical hemoglobins may be physiologically important in other ways. The presence of multiple hemoglobins with different surface characteristics may increase the overall solubility of hemoglobin in the red blood cell, allowing a greater oxygen carrying capacity per cell. The presence of hemoglobins with different isoelectric points will alter the intracellular pH due to the Donnan equilibrium across the erythrocyte membrane.

It has been shown in several fishes that multiple hemoglobins are the result of combinations of different globin chains which are the products of multiple copies of the genes for the α and β type chains. Amino acid composition and sequence data have shown this to be the case in several fishes. For example, in Fundulus heteroclitus (27) four different globin chains are combined to give six different hemoglobins. The possibility that some of the different globins present in fishes are due to post-translational modifications should

be considered however, since such modifications have been demonstrated to occur (N-terminal acetylation of the α chains) in these polypeptides.

It is thought that the different globin genes evolved by gene duplication and subsequent mutation. The α and β type globin genes of humans are each arranged in closely linked clusters on separate chromosomes. The structures of human (80) and mouse (81) globin genes indicate that extensive gene duplications have occurred in mammals. The globin gene clusters in mammals also contain pseudogenes which do not code for functional polypeptides due to frameshift mutations (80). Although direct evidence is lacking, it seems likely that the multiple globin genes of fishes also arose by gene duplications. The original divergence of the α and β globins probably arose from a tetraploid event (duplication of entire chromosomes) in an ancestral vertebrate (82). There is evidence that some fishes have undergone an additional tetraploid event more recently (83). This may account for the presence of two very different subtypes (anodal and cathodal) of α and β globin chains in Class II fishes.

From studies of several isoenzyme systems in salmonid and catostomid fishes, Allendorf (84) has shown that the degree of protein polymorphism is related to expression of duplicate genes in fishes. Proteins for which duplicate gene expression has been lost have a higher degree of polymorphism than those proteins for which duplicate gene expression is maintained. This relationship suggests that polymorphism is due to a loss of duplicate gene expression as a

result of random mutations. These mutations are neutral, since loss of gene expression for a particular isoenzyme apparently does not reduce the fitness of the individual. The greater retention of duplicate gene expression for some proteins may reflect a greater capacity of these proteins for random mutations without loss of function, as suggested by Allendorf. Alternatively, loss of expression of duplicate genes for these proteins may reduce the fitness of the individual so that loss of duplicate gene expression and polymorphism are selected against. Thus a high degree of polymorphism indicates that the multiple forms of a protein are not physiologically important, while a lack of polymorphism may suggest that the multiple forms provide a selective advantage to the fish.

Hemoglobins of trout—Numerous electrophoretic studies of hemoglobin show that salmonid fishes (salmon and trout) in general have both anodal and cathodal hemoglobins and thus represent the largest group of fishes belonging to Class II in the classification scheme described previously. Of this group of fishes, only the hemoglobins of rainbow trout have been well studied. As will be shown, the hemoglobins of rainbow trout are electrophoretically very similar to those of cutthroat trout. In this section the structural and functional properties of the hemoglobins of rainbow trout are reviewed. Relevant physiological studies and the limited information available regarding the hemoglobins of cutthroat trout are also discussed.

That rainbow trout contain multiple hemoglobins was first demonstrated by Buhler and Shanks in 1959 (85). They isolated three hemoglobins by moving boundary electrophoresis. Subsequent studies

using starch gel electrophoresis (41, 42, 86, 87, 88) indicated a greater number of hemoglobins, with the number of components resolved ranging from 4 to 11. Some of the differences in hemoglobin electrophoretic patterns may reflect differences between populations of rainbow trout. It is likely though that these variations are largely due to differences in resolution of the electrophoretic systems used and the formation of artifactual bands during electrophoresis. Artifactual bands may form in electrophoresis of hemoglobin by several means including oxidation of the heme irons, changes in heme ligation state, partial denaturation of the protein, and dissociation or aggregation of subunits. It is difficult to control for all these possibilities in a single electrophoretic system, even when they are recognized as experimental difficulties.

Most functional studies of rainbow trout hemoglobins have used the European rainbow trout, Salmo irideus. Four hemoglobins have been separated by starch gel electrophoresis and ion-exchange chromatography (89). These hemoglobins are designated Hb I, Hb II, Hb III, and Hb IV, with Hb I being the most cathodal on starch gels and eluting first from DEAE-Sephadex columns. Lau et al. (90) isolated four hemoglobins from the North American rainbow trout, Salmo gairdneri. The behavior of the four hemoglobins on starch gels and DEAE-cellulose columns is identical to that reported for S. irideus hemoglobins except that the relative proportions of Hb III and Hb IV are different. In S. irideus, Hb I and Hb IV are the major fractions, while in S. gairdneri Hb I and Hb III predominate. The corresponding hemoglobins from S. irideus and S. gairdneri have very similar oxygen equilibria (89, 90) and are antigenically indistin-

guishable (91). Thus it appears that the hemoglobins of these two species are very similar or identical. In this report no distinction is made between S. irideus and S. gairdneri, both fishes being referred to as rainbow trout.

Two of the hemoglobins from rainbow trout, Hb I and Hb IV, have been extensively studied. Both are tetramers (92) composed of two different globin chains (93) and have no subunits in common. The globin chains are designated α^{I}, α^{IV}, β^{I}, and β^{IV}. The complete amino acid sequences of the α and β chains of Hb I have been determined (94, 95) and the α^{IV} and β^{IV} chains have been partially sequenced (96). The N-terminal residue of both α^{I} and α^{IV} is acetylserine (97). A comparison of partial sequences of the α and β chains shows that Hb IV has greater sequence homology with α and β chains from carp than with those of Hb I. The large difference in primary structure between Hb I and Hb IV is reflected in the conformation of the two hemoglobins in solution. Circular dichroism studies (92) show that Hb IV has an α-helical content similar to that of human hemoglobin and undergoes a comparable conformational change upon oxygen binding. Hb I has a lower α-helical content which is unaffected by binding of oxygen. Antibodies raised against Hb I and Hb IV show no cross-reactivity (91), indicating that the surface properties of the two hemoglobins are very different.

The oxygen equilibrium curves of Hb I and Hb IV at alkaline pH are similar, but below pH 7.5 the affinity and cooperativity of oxygen binding for Hb IV decrease while these parameters for Hb I remain essentially constant between pH 8.5 and pH 6.8 (89). At pH

6.8, Hb I is completely saturated with oxygen in the presence of air (pO_2 = 155 mmHg) while Hb IV is 80% deoxygenated under these conditions. Thus Hb IV has a strong Root effect and at low pH the value of $n_{1/2}$ approaches 1, indicating non-cooperative oxygen binding.

The effect of nucleotides on the oxygen equilibria of these two hemoglobins is also different. In the presence of saturating levels of ATP the affinity and cooperativity of oxygen binding to Hb IV show a large decrease while the equilibrium curve for Hb I is essentially unchanged (98). Similar results are obtained with other organic phosphates, although the effect of GTP is not known. Heterotropic effects for Hb I are limited to small effects of inorganic ions, e.g., chloride, phosphate (98), and sodium (99). The effect of CO_2 on the oxygen equilibrium has not been determined for any of the hemoglobins of rainbow trout.

The oxygen equilibria of Hb I and Hb IV have very different temperature dependences (89). The oxygen affinity of Hb IV shows a large decrease as temperature is increased. The overall enthalpy of oxygenation for Hb IV is -14 kcal/mol at pH 8.5 and decreases as the pH is lowered (ΔH_{av} -7 kcal/mol at pH 7). In contrast, the oxygen eqiulibrium curve of Hb I is only slightly affected by temperature. The overall enthalpy of oxygenation is approximately zero and is independent of pH. More detailed studies (99, 100) show that the enthalpy of oxygenation for Hb I is dependent on the degree of saturation with oxygen. ΔH is approximately zero between Y = 0 and Y = 0.6, and approaches -8 kcal/mol at Y = 1.0.

There is no structural and little functional information available for Hb II and Hb III of rainbow trout. Limited oxygen

equilibrium data suggest that Hb II has properties similar to Hb I (89, 98) and that Hb III has properties similar to Hb IV (90).

Although it has been proposed (101, 102) that Hb IV (and Hb III) are important in oxygen secretion to the swimbladder in rainbow trout, salmonids lack a rete mirable associated with the swimbladder (61) and oxygen comprises less than 10% of the swimbladder gas, which is approximately 90% nitrogen in rainbow trout (103). Trout do have a choroid rete (65) and rely heavily on vision for prey capture, so the Root effect hemoglobins may be important in oxygen secretion to the eye in these fishes.

Trout are among the most active of teleost fishes. They frequently inhabit fast-moving streams and are able to swim rapidly to obtain prey and avoid predators. This capacity for high levels of activity is reflected in significant differences in respiratory function between rainbow trout and less active teleosts (104). As suggested by Powers and Edmundson (23) for catostomid fishes, the presence of pH insensitive cathodal hemoglobins such as Hb I (and Hb II) of rainbow trout may be important in oxygen transport during periods of high activity.

The red blood cells of rainbow trout contain both ATP and GTP. Measured values of the ATP/GTP ratio range from 9 (74) to 7.5 (105). ATP may be important in the regulation of blood oxygen affinity in response to hypoxia. Blood oxygen affinity increases significantly in rainbow trout acclimated to hypoxic water. There is conflicting evidence as to whether this is a result of changes in red blood cell ATP concentrations or in red blood cell pH. Tetens and Lykkeboe

(105) find that the ATP/Hb ratio decreases from 1.3 in normoxic (pO_2 = 150 mmHg) acclimated trout to 1.0 (pO_2 = 80 mmHg) and 0.5 (pO_2 = 50 mmHg) in hypoxic acclimated trout. They suggest that the increased blood oxygen affinity in hypoxic rainbow trout is accounted for by this decrease in red blood cell ATP. Nikinmaa and Soivio (106) find that this decrease in red blood cell ATP concentration is accompanied by a parallel decrease in hemoglobin concentration, both caused by an influx of water into the red blood cell, so that the ATP/Hb ratio is not significantly changed. They suggest that the influx of water leads to an increase in intracellular pH, so that the binding of ATP to hemoglobin is reduced and blood oxygen affinity is thereby increased.

Rainbow trout are not normally subject to large variations in temperature and cannot survive temperatures greater than about 25°C (107). Blood oxygen affinity and the red blood cell ATP/Hb ratio are not significantly different in fish acclimated to 5°C and 22°C (74). Indeed, all the standard hematological parameters of rainbow trout blood appear to be essentially constant throughout this temperature range (107). Changes in the relative proportions of hemoglobins (74, 108) and red blood cell ionic composition (72) have been reported to occur with acclimation to different temperatures. The significance of these changes has not been demonstrated though, and it appears that rainbow trout lack mechanisms to significantly increase oxygen transport at higher temperatures. This may be responsible for the relatively low upper temperature limit for rainbow trout.

The multiple hemoglobins of rainbow trout may be important in the development of this fish. Iuchi (88) found that the hemoglobins

of adult and larval rainbow trout have different electrophoretic patterns. The pattern observed for adult hemoglobin is similar to those observed by others (41, 42, 86, 87), but no bands migrating to the cathode (at pH 8.6) are present in the larval hemoglobin, and the anodal bands appear to have different mobilities than those of the adult. These apparent differences were confirmed by oxygen equilibrium measurements on crude hemolyzates of adult and larval fish (88). The adult hemolyzate displays a strong Root effect, as described previously by Giovenco et al. (87). Suprisingly, the larval hemolyzate, which lacks cathodal hemoglobins, has no Root effect and in fact, both the affinity and cooperativity of oxygen binding are independent of pH from pH 6.5 to pH 9.0. This is the only report in the literature of anodal hemoglobins from fish with pH independent oxygen equilibria. Thus it appears that larval rainbow trout have hemoglobins, distinctly different from those of adult fish, which may be important in serving different oxygen transport requirements.

In contrast to the hemoglobins of rainbow trout, very little is known regarding the hemoglobins of the cutthroat trout, Salmo clarki. Ronald and Tsuyuki (41) compared the hemoglobins of rainbow and cutthroat trout by starch gel electrophoresis. The hemoglobin pattern for both trout were essentially identical, both composed of four anodal and three cathodal bands at pH 8.5. Electrophoresis of globins showed that in both fishes the hemoglobins are derived from two different sets of globin chains, with the three most cathodal hemoglobins having no subunits in common with the other hemoglobins.

In a subsequent study, Braman et al. (42) performed a similar electrophoretic comparison of rainbow and cutthroat trout hemoglobins. The hemoglobins from rainbow trout were resolved into nine bands (1 cathodal and 8 anodal) by starch gel electrophoresis at pH 8.7. The pattern obtained for cutthroat trout hemoglobins was identical except for the presence of three additional cathodal bands. The apparent differences in hemoglobin electrophoretic patterns for cutthroat trout hemoglobins in these two studies may reflect differences in experimental techniques or may be due to the fact that Tsuyuki and Ronald used the anadromus coastal cutthroat trout while Braman et al. studied the freshwater inland cutthroat trout.

The question arises as to whether the additional hemoglobins in cutthroat trout are of physiological importance. There are no reports in the literature regarding functional properties of cutthroat trout hemoglobins. As described above however, a correlation may exist between the degree of polymorphism for a particular protein and the functional importance of the different forms of the protein. Hemoglobin polymorphism has been detected in one population of cutthroat trout (109), but this finding has not been confirmed in other populations of either cutthroat or rainbow trout (110, 111). The analysis of hemoglobin polymorphism in these fishes is complicated by the fact that rainbow and cutthroat trout frequently interbreed to yield viable hybrid fish. It is difficult to reliably distinguish hybrid fish from pure rainbow and cutthroat trout (43, 112, 113) and the rainbow x cutthroat hybrids have hemoglobin patterns similar to those presumed to be due to hemoglobin poly-

morphism in cutthroat trout (43). Thus it remains uncertain whether or not hemoglobin polymorphism occurs in cutthroat trout.

The primary goal of the current study is to isolate the hemoglobins of cutthroat trout and determine their oxygen binding properties. Particularly, we want to determine if the cathodal hemoglobins have properties different from those described for the cathodal hemoglobins of rainbow trout (Hb I and Hb II) which might be physiologically important in cutthroat trout.

MATERIALS AND METHODS

Materials—Trout were obtained from local streams by electroshock and held in tanks with circulating Logan River water at the Utah Water Research Laboratory, Logan. Normally, blood was sampled within one month of capture of fish. No deterioration in the physical condition of the fish was apparent during this holding period. No attempts were made to control the holding conditions.

Sephadex gels and Blue Dextran were obtained from Pharmacia. DEAE-cellulose (DE-52) was from Whatman. Poly(ethylene)imine (PEI) - cellulose sheets for TLC were from Merck. Ampholytes (Pharmalytes) and agarose (Agarose IEF) for isoelectric focusing were from Pharmacia. Starch for starch gel electrophoresis was from the Electrostarch Co., Madison, WI. Protein standards for isoelectric point and molecular weight determinations were from Pharmacia.

Nucleotides (ATP, ADP, AMP, GTP, GDP, and GMP), all >97% purity, were obtained from Sigma. Acetone for globin precipitations was triply distilled. Nitrogen gas for deoxygenation of hemoglobin was passed through hot copper turnings to remove trace oxygen and hydrated by bubbling through distilled water prior to use. All other chemicals were analytical grade and used without further purification. All buffers were prepared from either the free acid or free base and adjusted to the appropriate pH by titration with HCl or NaOH.

Isoelectric focusing and starch gel electrophoresis were performed on a Pharmacia Flat Bed Apparatus, FBE 3000, with an ISCO

Model 494 power supply. A homemade apparatus as described by Studier (114), modified by addition of a cooling plate, was used for vertical polyacrylamide gel electrophoresis. Agarose and polyacrylamide gels were prepared on plastic sheets (Gel Bond, Gel Bond PAG) from FMC. Hemoglobin solutions were concentrated by ultrafiltration with immersible membrane units (CX-10, molecular weight cutoff = 10,000) from Millipore. All spectroscopic measurements were made on a Cary 219 or Beckman DU-8 spectrophotometer.

Analysis of red blood cell nucleotides—Blood samples (0.5 ml) from three rainbow trout and three cutthroat trout were treated in parallel. Samples (obtained as described for purification of hemoglobins) were taken approximately 24 hr after capture of fish. Red blood cells were washed 3 times with 0.9% NaCl and lysed in three volumes of distilled water for 3 hr. After centrifugation at 27,000 x g for 1 hr, the supernatant was removed and 100% (w/v) TCA added to a final concentration of 10%. After 15 min on ice, the samples were centrifuged at 27,000 x g for 30 min to remove the protein precipitate. These acid extracts were freed of TCA by 3 extractions (2 vol each) with diethyl ether. Removal of acid was confirmed with pH paper. Samples were stored at -80°C for later analysis.

Nucleotides in the acid extracts were analyzed by thin layer chromatography on PEI-cellulose as described by Cashel et al. (115). After application of samples (20 ul), the sheet was dried under a stream of air and developed with 0.5 M potassium phosphate, pH 3.4. Spots were visualized under UV light and identified by comparison of mobilities relative to the pH front (R_{pHf}) to mobilities of known

standards (ATP, ADP, AMP, GTP, GDP, and GMP) run in parallel. Standards were prepared by dissolving 5 mg of each nucleotide in 5.0 ml of distilled water.

Identifications of nucleotides were confirmed by elution of spots from the chromatogram (116) and comparison of UV spectra. Spots were cut out and placed in 1.5 ml of 50 mM Tris, 0.7 M $MgCl_2$, pH 7.3. After 30 min with continuous shaking, the eluants were poured off and centrifuged at low speed for 5 min to remove suspended cellulose. A portion of the chromatogram with no sample applied was also eluted and the eluant used as the reference for spectrophotometry. Spectra were recorded from 230 nm to 320 nm.

Hemoglobin concentrations—Hemoglobin concentrations were determined by measuring the absorbance at 560, 576, and 630 nm. From these measured absorbances and the published extinction coefficients for human oxyhemoglobin, deoxyhemoglobin, and methemoglobin (117), the molar concentrations of the three hemoglobin species were calculated by solving the system of three simultaneous equations:

$$[6a] \quad A_{560} = \epsilon^{oxy}_{560}[oxy] + \epsilon^{deoxy}_{560}[deoxy] + \epsilon^{met}_{560}[met]$$

$$[6b] \quad A_{576} = \epsilon^{oxy}_{576}[oxy] + \epsilon^{deoxy}_{576}[deoxy] + \epsilon^{met}_{576}[met]$$

$$[6c] \quad A_{630} = \epsilon^{oxy}_{630}[oxy] + \epsilon^{deoxy}_{630}[deoxy] + \epsilon^{met}_{630}[met]$$

Since the extinction coefficients of methemoglobin are pH dependent, a different set of equations is required for each pH at

which concentrations are to be determined. A computer program was written to choose the proper methemoglobin extinction coefficients for any pH from 6.0 to 9.0 (in 0.1 pH unit increments). The appropriate system of equations was then solved for the concentrations of oxy-, deoxy-, and methemoglobin by the Gauss-Jordan iterative method (118). The percentage of oxyhemoglobin was calculated based on the total hemoglobin concentration and based on [oxy] + [deoxy] = 100%. This second value is the percent oxyhemoglobin corrected for methemoglobin and was used in all measurements of oxygen binding by hemoglobin (usually expressed as Y, the fractional saturation with oxygen). Unless specifically stated, all hemoglobin concentrations are expressed on a heme basis. Concentrations expressed in terms of mg/ml are based on a molecular weight of 16,000/heme.

Starch gel electrophoresis—Starch gel electrophoresis of hemoglobins was performed essentially as described previously (42, 111) except that samples were applied as oxyhemoglobin rather than as cyanomethemoglobin and KCN and DTT were omitted from the gels. The electrode buffer (0.18 M Tris, 0.1 M boric acid, 4 mM EDTA, pH 8.7) was diluted 1:10 for preparation of the gel. Gels (20 x 14 x 0.5 cm) were electrophoresed for 4 hr at 300 V, sliced, and stained with Coomassie Blue or o-dianisidine, and destained in methanol:water: acetic acid (5:5:1).

Analytical isoelectric focusing—Agarose gels (1%) containing 12% sorbitol were prepared as described by Pharmacia (119). Gel dimensions were 21 x 11 x 0.1 cm and the separation distance was 11

cm. Different ampholyte intervals were mixed to obtain optimal separations. Unless otherwise noted, a mixture of pH 3-10 (0.5 ml), pH 6.5-9 (1.0 ml), and pH 8-10.5 (0.4 ml) ampholytes was used. Samples (5-50 ug in 5-25 ul) were applied to the center of the gel on small pieces of filter paper. Gels were focused for 90 min at 15 W constant power. The cooling plate was supplied with water at 10°C.

Focused gels were fixed in 10% TCA, 5% sulfosalicylic acid for 30 min. TCA and ampholytes (which interfere with staining) were removed by two washes (30 min each) with 35% ethanol, 10% acetic acid. Gels were then dried and stained for 15 min with 0.2% Coomassie Blue, 35% ethanol, 10% acetic acid. Gels were destained for 15-30 min in the dye solvent and finally dried with a hair dryer.

In some cases, samples were converted to methemoglobin or cyanomethemoglobin prior to focusing. The hemoglobin (50-100 uM) in 5 mM Tris, 0.1 M NaCl, pH 7.7 was oxidized by addition of 1.2 equiv/heme of potassium ferricyanide in the same buffer. The ferricyanide solution was added dropwise with stirring and the resulting brown methemoglobin solution immediately applied to a column (0.9 x 25 cm) of Sephadex G-25 equilibrated with 5 mM Tris, 0.1 M NaCl, pH 7.7 to separate the hemoglobin from product ferrocyanide and excess ferricyanide ions. The extent of oxidation of the hemoglobin was determined by measuring the absorbance at 560, 576, and 630 nm and calculating the concentrations of oxy-, deoxy-, and methemoglobin as described above. The cyanomet derivative was prepared from methemoglobin by addition of 3 equiv/heme of KCN in 5 mM Tris, 0.1 M NaCl,

pH 7.7. All these procedures were performed at 4-8°C.

Determination of isoelectric points—Isoelectric points of the hemoglobin components were determined by isoelectric focusing in agarose gels. Conditions were as described for analytical isoelectric focusing with the following changes as recommended by Pharmacia (120): (a) the coolant water was maintained at 18°C to give a temperature in the gel of 24 +/- 1.5°C; and (b) during focusing, 1 M NaOH was placed in the buffer vessels and the lid of the apparatus was sealed with tape to minimize absorption of CO_2 by the gel. Isoelectric points were calculated by comparison of the positions of focused bands to the positions of marker proteins of known pI (Pharmacia high pI calibration kit).

Determination of molecular weight—The molecular weight of unfractionated cutthroat trout hemoglobin was estimated by gel exclusion chromatography. A 1.5 x 85 cm column of Sephadex G-100 Superfine was prepared and equilibrated with 5 mM Tris, 0.1 M NaCl, pH 8.2. A sample of whole (unfractionated) hemoglobin was dialyzed against this buffer and 0.5 ml (15 mg hemoglobin) was added to 0.2 ml of Blue Dextran (4 mg/ml) also in this buffer. This solution was applied to the column and eluted at a flow rate of 5 ml/hr. Fractions (1.0 ml) were collected and the absorbance at 415 nm measured. The column was equilibrated and run at 5°C.

The column was calibrated by chromatography of proteins of known molecular weight. A calibration curve was constructed by plotting K_{av} (the available diffusion coefficient):

[7] $$K_{av} = \frac{V_e - V_o}{V_t - V_o}$$

where:

V_e = elution volume of the standard

V_o = void volume of the column (V_e for Blue Dextran)

V_t = total volume of the gel (150ml)

versus the log of the molecular weight for the standards. Molecular weight standards were ribonuclease (13,700), chymotrypsinogen (25,000), ovalbumin (43,000), and albumin (67,000).

Purification of cutthroat trout hemoglobins—Fish were anesthetized with tricaine methane sulfonate (16 ppm) plus quinaldine (3.5 ppm) and blood collected from the caudal vein with acid-citrate-dextrose (121) as anticoagulant. Blood from several fish was pooled for purification of hemoglobins. Samples were packed in ice for transport and all further purification steps (except isoelectric focusing) were performed in a cold room at 4-8°C.

Cells were washed three times with 0.9% NaCl and lysed in 5 vol of 1 mM Tris, pH 8.2 for 3 hr. Unlysed cells and debris were removed by centrifugation at 27,000 x g for 1 hr.

The hemoglobin was separated from nucleotides and other small molecules by chromatography on Sephadex G-25 (122). After addition of 1/20 vol of 0.1 M Tris, 2.0 M NaCl, pH 8.3, the crude hemolyzate was applied to a column (2.5 x 60 cm) of Sephadex G-25 equilibrated with 5 mM Tris, 0.1 M NaCl, pH 8.2, and eluted with this buffer. The flow rate was 35 ml/hr, 3.0 ml fractions were collected, and the absorbance at 254 nm measured.

The whole (unfractionated) hemoglobin eluted from the G-25 column was dialyzed overnight against 0.1 M Gly, pH 8.2. The dialyzed hemoglobin was applied to a column (1.5 x 30 cm) of DEAE-cellulose equilibrated with this buffer. The hemoglobins were eluted with a NaCl step gradient consisting of the starting buffer plus 0, 10, 25, and 100 mM NaCl. The flow rate was 15 ml/hr, 1.0 ml fractions were collected, and the absorbance at 540 nm measured.

The fractions eluting with the starting buffer and with 100 mM NaCl were further purified by isoelectric focusing in Sephadex G-200. Prior to focusing, the fraction eluting with the starting buffer was concentrated to 10-12 ml and the 100 mM NaCl fraction was dialyzed against 0.1 M Gly, pH 8.2 to remove the NaCl. The gel bed (23 x 23 x 0.5 cm) was prepared as follows: 12 g G-200 was swollen in distilled water and then washed in a Buchner funnel with 8 L of distilled water. To the partially dried gel were added 12 ml of ampholytes and enough distilled water to give a gel that would not hold air bubbles. The gel was degassed for 30 min and spread on the Pharmacia gel plate. Portions of the gel at each end of the plate were removed and the pre-soaked electrode strips (0.1 M H_2SO_4, cathode and 0.2 M ethylene diamine, anode) were inserted.

Samples were prepared for focusing by adding approximately 0.7 g of dry Sephadex G-200 to 10-12 ml of the hemoglobin solution (approximately 10 mg/ml) in 0.1 M Gly, pH 8.2 to make a gel of the same consistency as the gel bed. After pre-focusing for 45 min at 8 W, a 1 cm wide section of the gel was removed from the center of the plate and replaced with the sample gel. The gel was then

focused for 6 hr at 25 W. The cooling plate was supplied with water at 4°C.

After focusing, a paper print (23 x 2 cm) of the gel was made and stained with Coomassie Blue as described by Radola (123). The focused bands were removed from the gel bed and a minimal amount of 5 mM Tris, 0.1 M NaCl, pH 7.7 added to give a gel that could be poured. This gel was poured onto a column (1.5 x 15 cm) of Sephadex G-25 equilibrated with the same buffer and the hemoglobin eluted with this buffer. The hemoglobin was then dialyzed overnight against 5 mM Tris, 0.1 M NaCl, pH 7.7 to remove remaining ampholytes.

The optimal ampholyte mixtures for preparative isoelectric focusing of the partially purified hemoglobins were determined by analytical scale (gel dimensions: 23 x 11 x 0.2 cm) isoelectric focusing in Sephadex as described by Radola (123). Various mixtures of pH 3-10, pH 5-8, pH 6.5-9, and pH 8-10.5 ampholytes were tested.

Oxygen equilibria of cutthroat trout hemoglobins—The oxygen equilibria of the isolated hemoglobins were studied by tonometry (124, 125). The tonometer used (Fig. 4) was modified from the design of Allen et al. (126). The tonometer consists of: (a) a 1 ml, 1.0 cm path length quartz cell; (b) an equilibration chamber; (c) a gas reservoir; (d) a stopcock through which the tonometer can be alternately flushed with gas and evacuated; (e) a ground glass joint for connection to a rotating-drive motor (from a roto-vap); and (f) a tube used to introduce the sample and to add known volumes of gas by means of a gas-tight syringe (through a serum cap fitted over the tube). The internal volume of the tonometer is 266 ml.

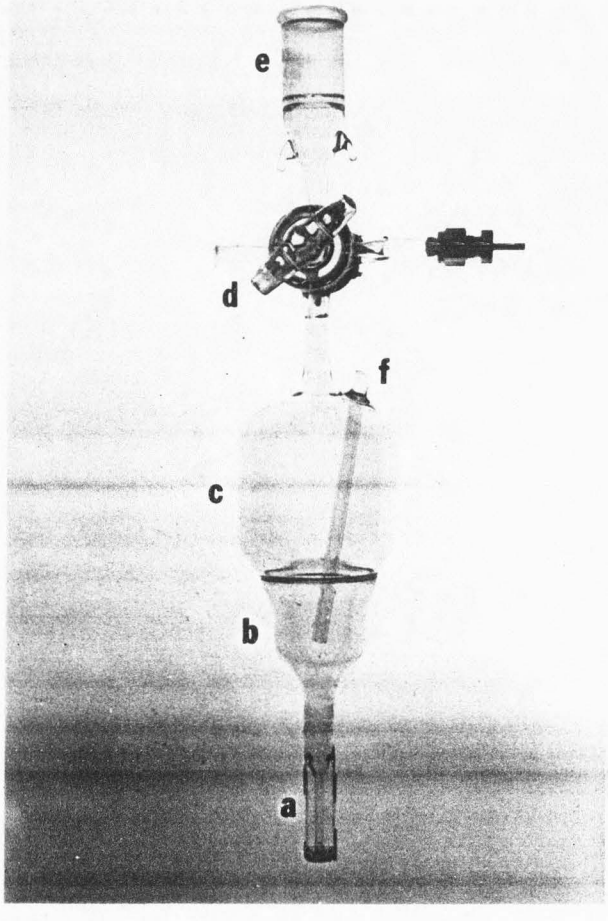


FIG. 4. **The tonometer used to determine oxygen equilibrium curves.** a, 1 ml quartz cell; b, equilibration chamber; c, gas reservoir; d, stopcock for flushing with gas and evacuating; e, joint for connection to rotating-drive motor; f, addition tube.

The procedure for determination of an oxygen equilibrium curve was as follows: (1) Approximately 1 ml of the hemoglobin solution was added to the tonometer by means of a syringe fitted with plastic tubing. The tonometer was tilted horizontally so that the solution flowed into the equilibration chamber and was then placed in a constant temperature water bath. The tonometer was then rotated for several minutes to equilibrate the sample at the bath temperature, removed from the bath, tilted vertically, and placed in the sample cell of the spectrophotometer (supplied with circulating water from the constant temperature bath), and the initial absorbance reading taken; (2) The tonometer was then returned to the bath for deoxygenation. The tonometer was first flushed with nitrogen and the tube (f) closed with a serum cap. The tonometer was then evacuated, filled with nitrogen, and evacuated. The gas inlet and vacuum lines were disconnected and the tonometer rotated for several minutes. The lines were then reconnected, the tonometer filled with nitrogen and evacuated twice more, and again rotated in the bath. The tonometer was then removed from the bath, placed in the spectrophotometer, and the deoxygenated absorbance reading taken; (3) Oxygen was introduced into the evacuated tonometer by addition of a known volume of air from a gas-tight syringe through the serum cap. After each addition, the tonometer was equilibrated and the absorbance read.

The partial pressure of oxygen in the tonometer after each addition of air was calculated from the equation:

$$[8] \qquad pO_2 = \frac{P_b \times X_o \times V_i}{V_t}$$

where:

pO_2 = partial pressure of oxygen in the tonometer

P_b = barometric pressure

X_o = mole fraction of oxygen in air (0.2095)

V_i = volume (in ml) of air introduced

V_t = volume of the gas phase in tonometer (265ml)

Normally, 8-10 additions of air (2-30 ml each) were made after the deoxygenated reading and then the final reading taken with the tonometer open to the atmosphere. In some cases a further reading was made with the tonometer filled with oxygen at the barometric pressure. The fractional saturation with oxygen (Y) at each step was calculated from the equation:

$$[9] \qquad Y = \frac{[oxy]}{[oxy] + [deoxy]}$$

using the hemoglobin concentrations calculated as described above.

Oxygen equilibrium data were analyzed in terms of the Hill plot by plotting log [Y/(1-Y)] versus log pO_2. The experimental points were fitted to the Adair equation for four-step oxygenation of hemoglobin as described by Imai (127). Values for the Hill coefficient, $n_{1/2}$, and the log of the partial pressure of oxygen at Y = 0.5, log $p_{1/2}$, were obtained from the theoretical curve fitted to the data.

Buffers for oxygen equilibrium measurements were all 50 mM with respect to the buffer component and 0.1 M in NaCl. The following buffers were used:

Buffer	pH
Bis-tris	6.20
Bis-tris	6.48
Bis-tris	6.80
Tes	7.03 (7.13 at 10°C)
Tes	7.11
Tes	7.20 (7.10 at 30°C)
Tes	7.42
Taps	7.81
Taps	8.00

The buffers were equilibrated at 20°C and pH adjusted on a Radiometer Model 26 pH meter. The pH 7.03 and pH 7.20 Tes buffers were prepared for oxygen equilibrium measurements at 10°C and 30°C respectively. The pH at these temperatures were calculated using the temperature coefficient for Tes, $\Delta pKa/^{\circ}C = -0.020$ (128). Except for temperature studies, all oxygen equilibria were determined at 20°C.

Hemoglobin solutions for tonometry were prepared by adding 50-200 ul of the purified hemoglobin in 5 mM Tris, 0.1 M NaCl, pH 7.7 to 1.0 ml of one of the buffers described above. The final hemoglobin concentration was 5-30 uM. When necessary, the purified hemoglobin solution was first concentrated to attain the required concentration for absorbance measurements in the tonometer.

The effect of ATP and GTP on the oxygen equilibria was studied at pH 7.1 and 20°C. Stock solutions (20 mM) of ATP and GTP in 0.1 M Tes, 0.1 M NaCl, pH 7.1 were prepared immediately prior to use and purity of the nucleotides checked by thin layer chromatography on PEI-cellulose. Addition of 10-25 ul of the stock nucleotide solution to the hemoglobin solution (1.05-1.20 ml) gave a nucleotide concentration of 200-500 uM and a nucleotide to heme ratio of about 20.

The effect of temperature on the oxygen equilibria was studied

from 10°C to 30°C at pH 7.1. For measurements at 15°C and 25°C, the pH 7.11 (at 20°C) Tes buffer was used. The calculated pH values of this buffer at these two temperatures are 7.16 and 7.06, respectively. The overall enthalpy of oxygenation, (ΔH_{av}), for each hemoglobin was calculated by the modified van't Hoff equation (eq. [5]). The values of log $p_{1/2}$ from the Hill plots were plotted versus the reciprocal of the absolute temperature. The slope of the line equals 2.3 R ΔH_{av}. All values of ΔH_{av} reported are corrected for the heat of solution of oxygen (-3.1 kcal/mol at 20°C) and expressed per mol O_2.

Preparation of globins—Globins were prepared from the purified hemoglobins by precipitation in acidic acetone as described by Chernoff and Pettit (129). Hemoglobin samples were first dialyzed exhaustively (48 hr) against distilled water. For precipitation, the dialyzed hemoglobin (1-5 mg/ml) was added dropwise to 25-30 vol of cold (-20°C to -10°C) acidic acetone (3 ml of 2 N HCl per liter) with mixing on a vortex mixer. After 10 min at -20°C with intermittent mixing, the solution was centrifuged at 12,000 x g for 20 min (at -10°C). The globin precipitate was washed with two portions of cold acetone and dissolved in 1-2 ml of distilled water. After centrifugation at 12,000 x g for 10 min, the soluble globin was lyophilized and stored at -80°C.

Separation of globin chains—Globin chains were separated by two methods: (a) electrophoresis in 12% polyacrylamide gels containing 6 M urea, 5% acetic acid, and 2% Triton X-100 (130); and (b) isoelectric focusing in 2% agarose gels containing 8 M urea, 2%

Triton X-100, and 1% 2-mercaptoethanol (131).

Slab gels (14 x 12 x 0.1 cm) for polyacrylamide gel electrophoresis were prepared and pre-electrophoresed as described (130) except that the amounts of Temed and ammonium persulfate were doubled, and in some gels, Triton X-100 was omitted. The running pH of the gels was 2.4. Approximately 10 ug of each globin in 3-5 ul of the sample buffer (6 M urea, 8% acetic acid, 8% 2-mercaptoethanol) were placed in the sample wells and the gel electrophoresed for 15 hr at 200 V. During the run, the current dropped from 25-30 mA to 10-15 mA. Gels were stained overnight in 0.2% Coomassie Blue, 35% ethanol, 10% acetic acid, and destained in the dye solvent.

Agarose gels for isoelectric focusing were prepared as described (131) except that a mixture of ampholytes (1.0 ml pH 5-8, 0.5 ml pH 3-10, and 0.4 ml pH 6.5-9) was used. Gel dimensions were the same as for isoelectric focusing of hemoglobins. After prefocusing for 40 min at 10 W, samples (the same as for electrophoresis of globins) were applied to small wells cut in the center of the gel. Gels were then focused for 90 min at 10 W and the gels fixed and stained as described (131).

RESULTS

Analysis of red blood cell nucleotides—Thin layer chromatography of the red blood cell acid extracts from cutthroat and rainbow trout showed the presence of 5 different nucleotides in the cutthroat trout extract and 3 different nucleotides in the rainbow trout extract. There was no variation in the chromatographic pattern for extracts from three individual fish of each species.

Comparison of the mobilities of the nucleotides in the acid extracts to those of the standards (Table I), shows that the cutthroat trout extract contains both adenosine and guanosine nucleotides while the rainbow trout extract contains only adenosine nucleotides. No spots with mobilities corresponding to GTP, GDP, or GMP could be observed in the rainbow trout extracts with sample loads up to 100 ul (the highest load at which spots could be resolved).

The identifications assigned to the nucleotides in the acid extracts by comparison of mobilities were confirmed by elution of spots from the chomatogram and comparison of UV spectra. The spectra for the spots from the cutthroat trout extract with mobilities corresponding to ATP and GTP are compared to the spectra for the standard spots in Fig. 5 and Fig. 6. The spectral data for these four eluted spots are summarized and compared to literature values (132) for ATP and GTP in Table II.

This analysis shows that both ATP and GTP are present in the red blood cells of cutthroat trout. The red blood cells of rainbow trout also contain ATP, but no GTP (or any guanosine nucleotides)

TABLE I

Thin layer chromatography of nucleotides on PEI-cellulose

The chromatogram was developed with 0.5 M potassium phosphate, pH 3.4. Mobilities of spots in the acid extracts are average values for three individual samples of each extract. Identities were assigned by comparison of mobilities with standards and confirmed by elution of spots and comparison of UV spectra, except for the spot identified as AMP + GMP (identity assigned by comparison of mobilities only).

Sample		Mobility (R_{pHf})	Identity
Standards:	AMP	0.84	
	GMP	0.76	
	ADP	0.69	
	GDP	0.49	
	ATP	0.38	
	GTP	0.20	
Cutthroat trout red blood cell acid extract		0.79	(AMP + GMP)
		0.67	ADP
		0.52	GDP
		0.37	ATP
		0.19	GTP
Rainbow trout red blood cell acid extract		0.82	AMP
		0.67	ADP
		0.39	ATP

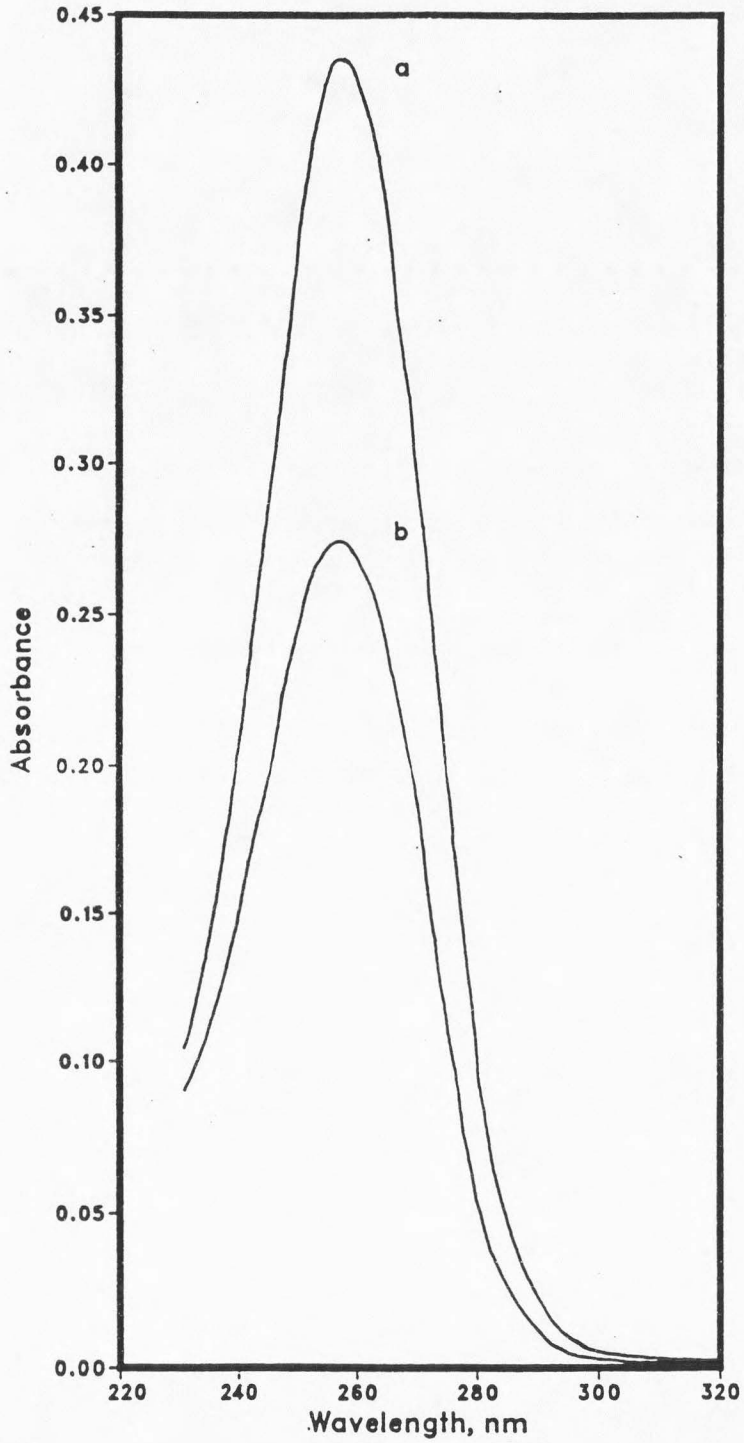


FIG. 5. **UV spectra of spots eluted from PEI-cellulose chromatogram.** Spots were eluted in 1.5 ml 50 mM Tris, 0.7 M $MgCl_2$, pH 7.3 and the spectra taken directly. a, ATP standard; b, R_{pHf} = 0.37 spot from cutthroat trout red blood cell acid extract.

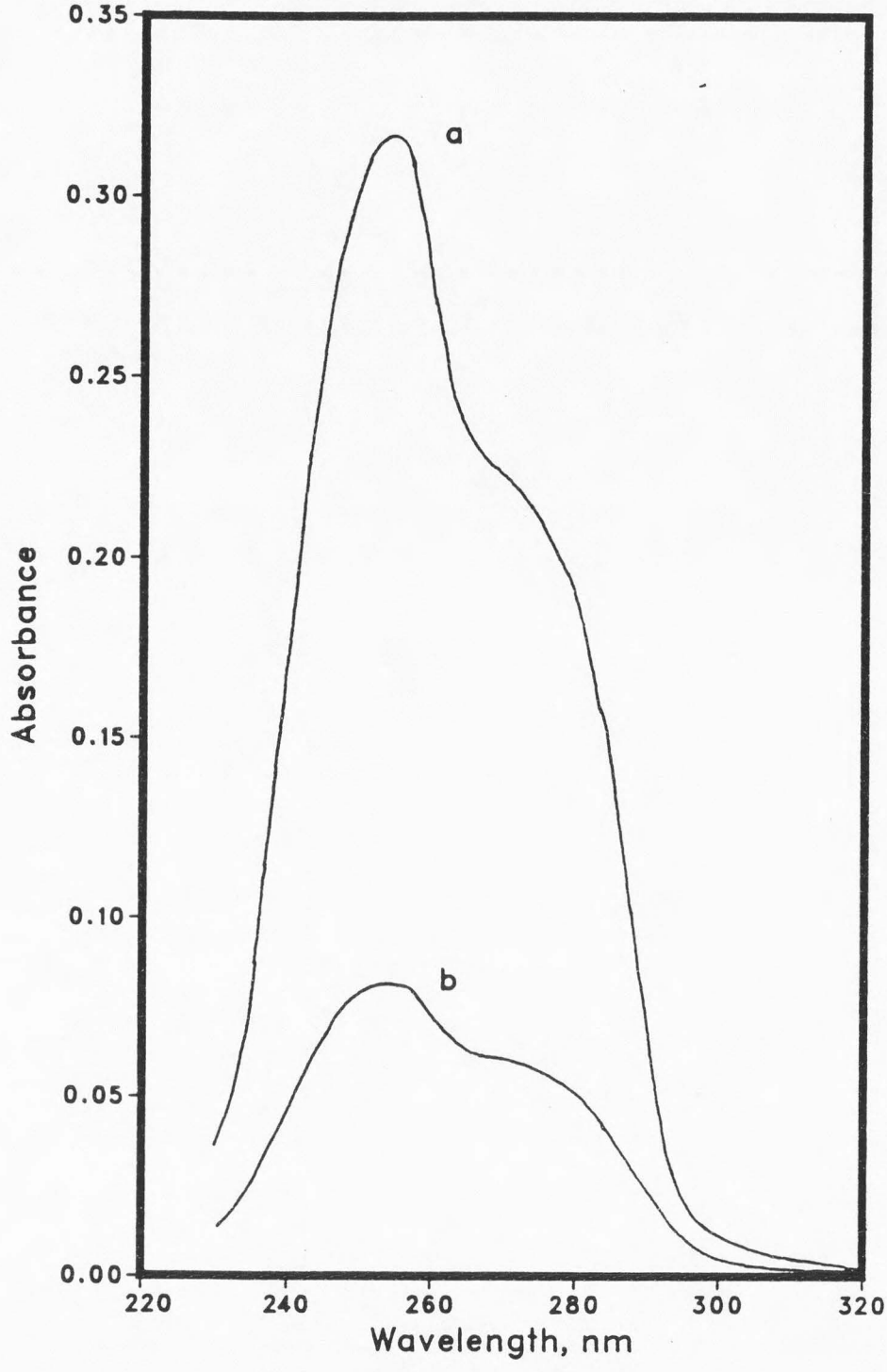


FIG. 6. **UV spectra of spots eluted from PEI-cellulose chromatogram.** Spots were eluted in 1.5 ml 50 mM Tris, 0.7 M $MgCl_2$, pH 7.3, and the spectra taken directly. a, GTP standard; b, R_{pHf} = 0.19 spot from cutthroat trout red blood cell acid extract.

TABLE II

UV spectral data for spots eluted from PEI-cellulose chromatogram

Values for extract and standard spots are for the spectra in Figs. 5 and 6. Literature values are from reference 132.

	λ_{max}, nm	A_{250}/A_{260}	A_{280}/A_{260}
ATP			
extract spot	258	0.89	0.22
standard spot	258	0.86	0.23
literature	259	0.80	0.15
GTP			
extract spot	255	1.1	0.67
standard spot	254	1.14	0.68
literature	253	1.17	0.66

could be detected by the method used. Assuming an equal yield for ATP and GTP in the extraction and elution procedures and no difference in degradation of the nucleotides during storage (60 days at -80°C) the molar ratio of ATP to GTP in the red blood cells of cutthroat trout can be estimated from the observed spectra for the eluted spots and the extinction coefficients for ATP and GTP (132). This calculation gives an ATP/GTP ratio of 3.

Purification of cutthroat trout hemoglobins—The purification scheme for cutthroat trout hemoglobins was designed to yield relatively large quantities of purified hemoglobins in a form suitable for oxygen equiliblium measurements. Most important in this regard is that oxidation to methemoglobin during the purification procedure be kept to a minimum. To accomplish this goal, the hemoglobin was kept at 4-8°C and at pH 7.7-8.2 during the purification (except during isoelectric focusing). Under these conditions essentially all of the hemoglobin is present as oxyhemoglobin and the rate of heme oxidation is minimized (133). In addition, every attempt was made to accomplish purification in the shortest possible time. Since the primary interest was in studying the four cathodal hemoglobins, emphasis was placed on the purification of these components.

The purification scheme is outlined in Fig. 7, and consists of essentially five steps after collection of the blood: (I) separation of red blood cells from serum, white blood cells, and anticoagulant by repeated washing and centrifugation; (II) hypotonic lysis of red blood cells to release the cell contents followed by centrifugation to remove cell membranes; (III) separation of hemoglobin from nucle-

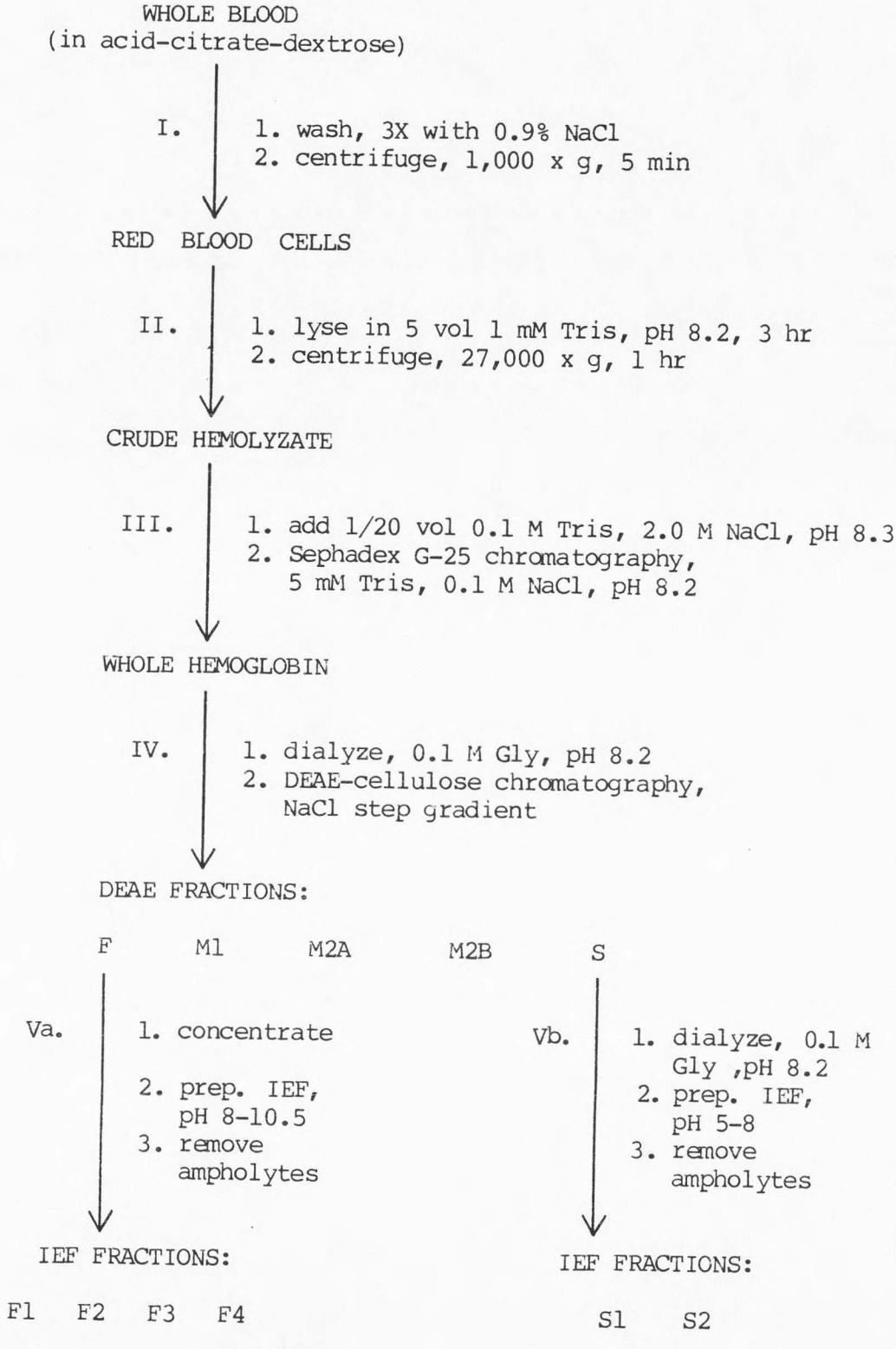


FIG. 7. **Purification scheme for cutthroat trout hemoglobins.**

otides and other small molecules by Sephadex G-25 chromatography; (IV) separation of the whole hemoglobin into five fractions by ion-exchange chromatography on DEAE cellulose; and (V) further purification of two of the fractions from the DEAE column by preparative isoelectric focusing in a horizontal bed of Sephadex G-200.

Washing of the red blood cells was performed by the standard method for blood samples and yielded 3-4 ml of packed cells from 10 ml of whole blood. Cells were lysed in dilute buffer rather than distilled water as commonly reported in order to maintain a pH of about 8 and thereby minimize oxidation to methemoglobin during lysis.

In order to remove nucleotides and other small molecules which may act as allosteric modulators of hemoglobin, the hemolyzate was chromatographed on a column of Sephadex G-25 equilibrated with 5 mM Tris, 0.1 M NaCl, pH 8.2. Rather than dialyzing the hemolyzate against the elution buffer, a concentrated Tris/NaCl solution was added and the hemolyzate applied immediately to the column. Separation of hemoglobin from nucleotides in the hemolyzate was observed by monitoring the elution profile at 254 nm (Fig. 8). Hemoglobin fractions with an absorbance at 254 nm greater than 2.0 were pooled for further purification. The pooled fractions from the G-25 column (whole hemoglobin) had an A_{254}/A_{576} ratio of 1.8-1.9, compared to 2.3-2.4 for the crude hemolyzate.

Initial fractionation of cutthroat trout hemoglobin was accomplished by ion-exchange chromatography. In order to optimize the separation of the hemoglobins on the column, several chromatographic systems were tested. Different column resins (DEAE-Sephadex, DEAE-

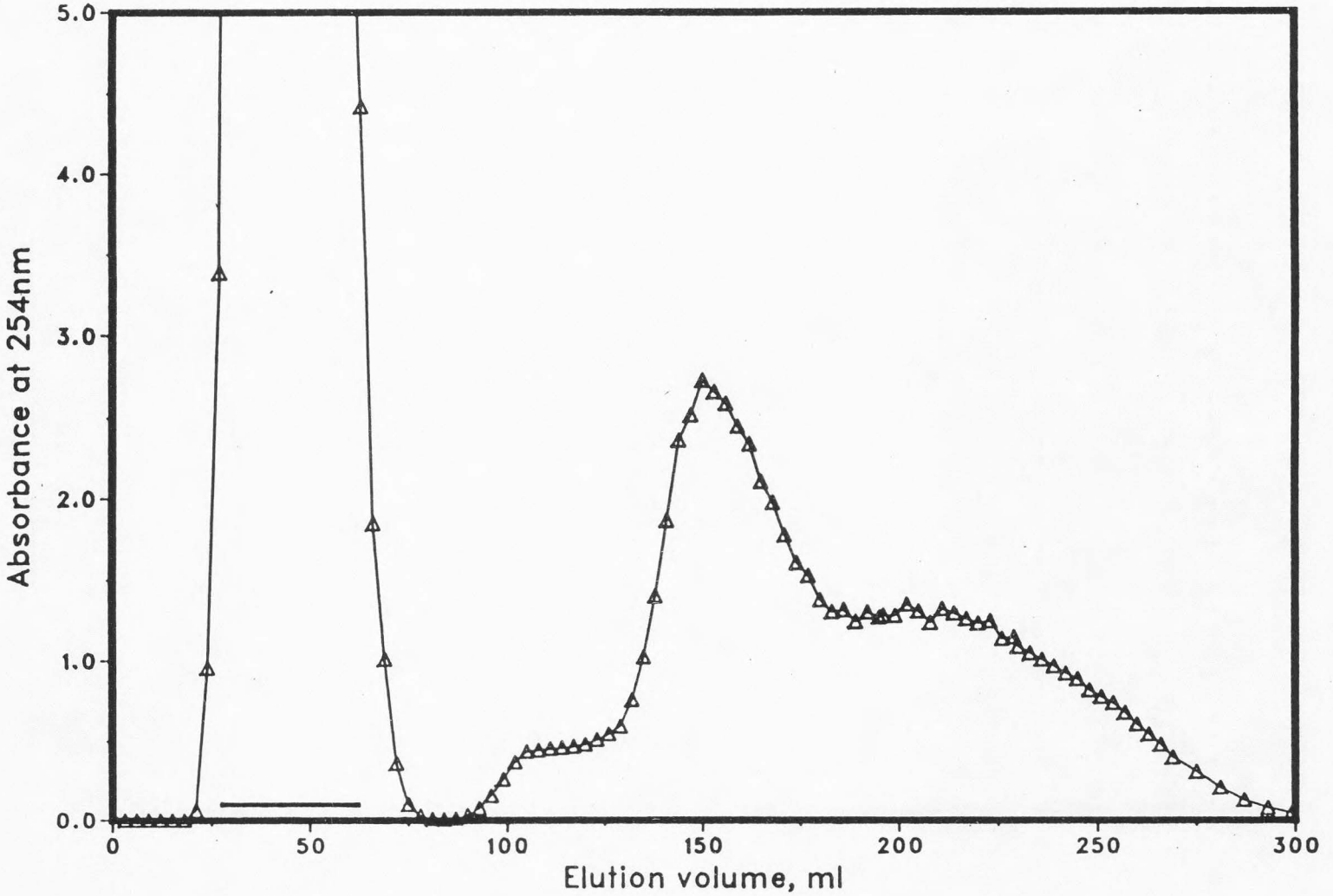


FIG. 8. **Chromatography of crude hemolyzate on Sephadex G-25.** Sample volume was 17 ml and 530 mg hemoglobin was applied. Conditions as described in Materials and Methods. The horizontal bar indicates the hemoglobin fractions pooled for further purification.

cellulose, CM-Sepharose CL-6B, CM-cellulose), buffers (phosphate, Tris, Gly), and elution systems (linear pH gradients, linear and step NaCl gradients) were employed in these studies. The system finally chosen, elution with a step NaCl gradient in 0.1M Gly, pH 8.2 on DEAE cellulose, provides good separation of the hemoglobins with essentially no dilution in a relatively short time.

A typical elution profile for the DEAE-cellulose column is shown in Fig. 9. After elution of the first fraction (fraction F) with the starting buffer, the buffer was replaced with starting buffer containing 10 mM NaCl and elution continued. A single peak (fraction M1) was eluted with this buffer, which was then replaced with starting buffer containing 25 mM NaCl. Two partially separated peaks (fractions M2A and M2B) were eluted with this buffer. Increasing the NaCl concentration to 100 mM resulted in elution of the remaining hemoglobin in a single peak (fraction S).

The purity of the fractions eluted from the DEAE-cellulose column was assessed by analytical isoelectric focusing (Fig. 10). The lighter, more cathodal bands visible for some samples on these gels are probably due to oxidation to methemoglobin during IEF, and not due to contamination with other hemoglobins. Fractions M1, M2A, and M2B each focused as a single band and were not further purified. Fraction F was resolved into four well separated bands, while fraction S focused as a broad band which appears to contain several different bands. These assessments of purity were confirmed by starch gel electrophoresis (Fig. 11), except that fraction M2A could not be visualized in the starch gel due to its low concentration. In each run of the purification procedure, either fraction F or

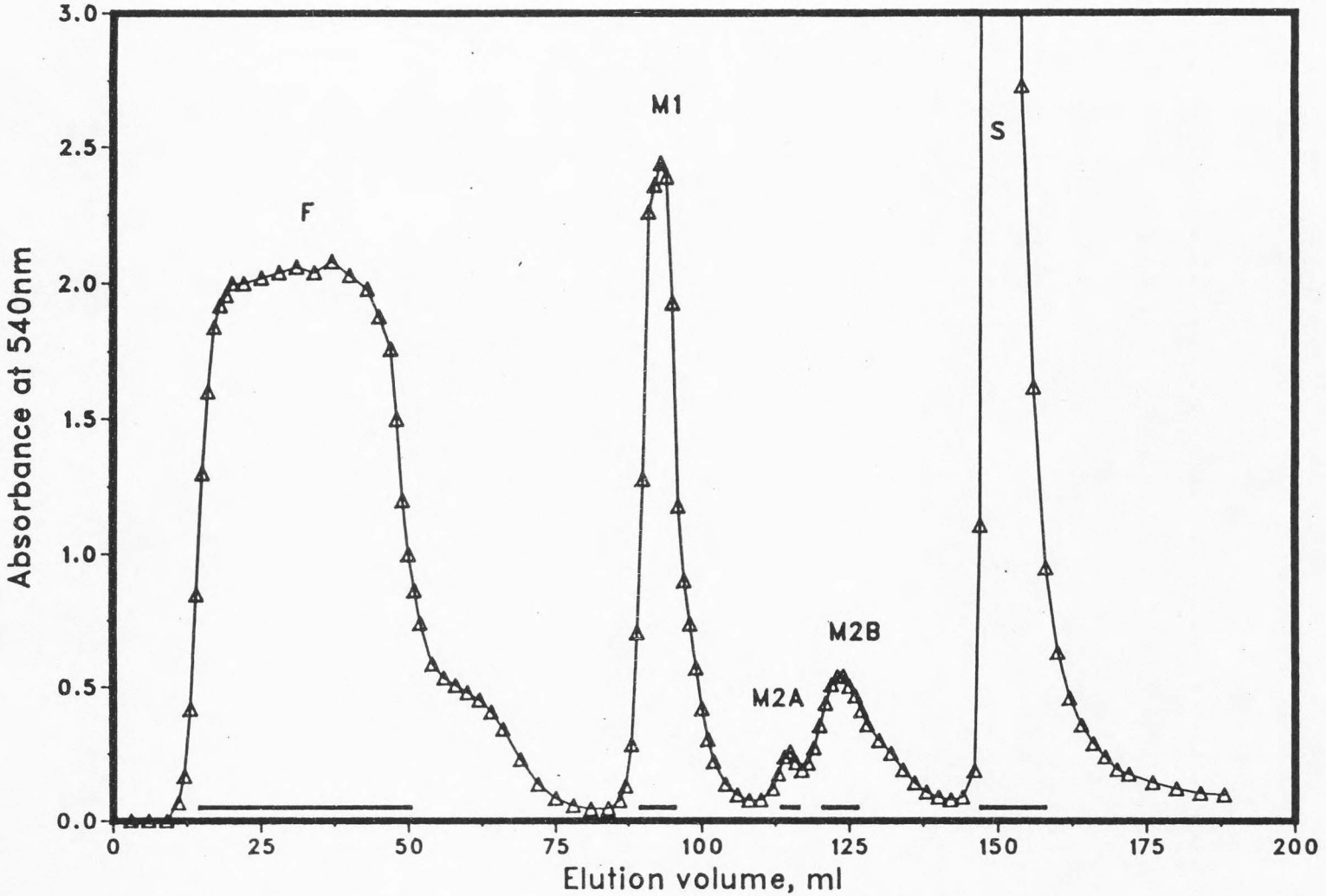


FIG. 9. **DEAE-cellulose chromatography of cutthroat trout hemoglobins.** The sample (470 mg Hb in 36 ml 0.1 M Gly, pH 8.2) was applied and fractions eluted with this buffer + 0 mM NaCl, F; + 10 mM NaCl, M1; + 25 mM NaCl, M2A and M2B; + 100 mM NaCl, S. Horizontal bars indicate the fractions pooled.

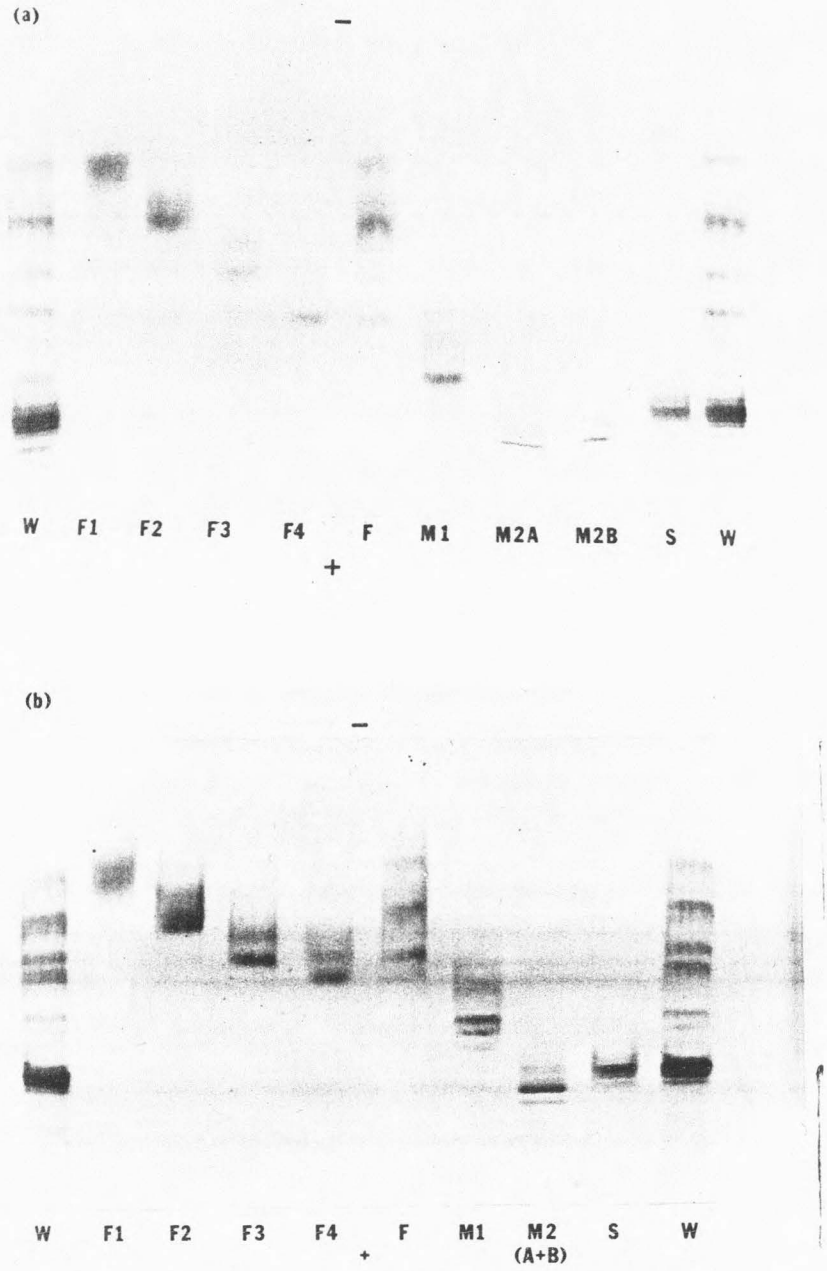


FIG. 10. **Analytical isoelectric focusing of cutthroat trout hemoglobins.** Results of two separate isolations are shown in (a) and (b). Samples in (a) are: W, whole hemoglobin from the Sephadex G-25 column; F, M1, M2A, M2B, and S, fractions from the DEAE column; F1, F2, F3, and F4, fractions from preparative isoelectric focusing of fraction F. Samples in (b) are the same as for (a) except that fractions M2A and M2B were not separated in this case. Focusing conditions (as described in Materials and Methods) were the same for both gels. The cathode is at the top of each gel.

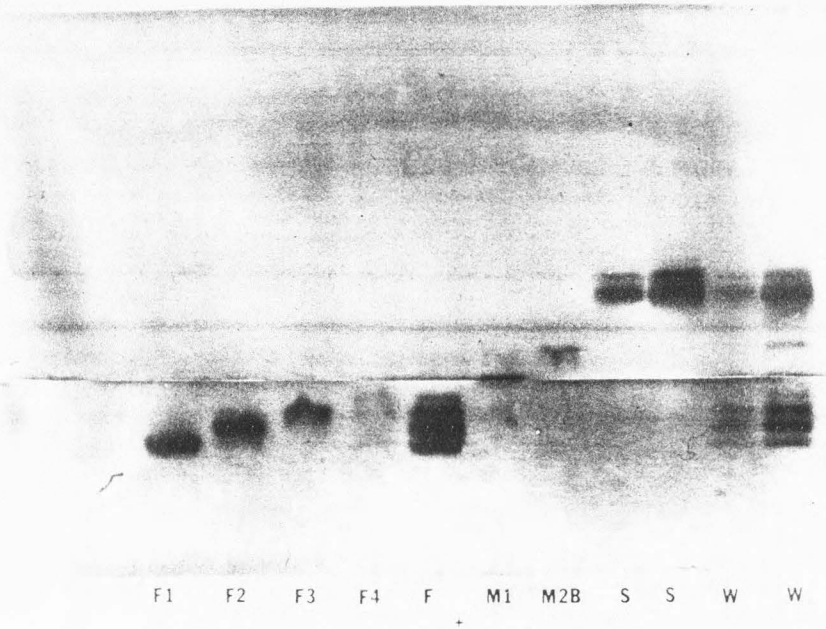


FIG. 11. **Starch gel electrophoresis of cutthroat trout hemoglobins.** Samples are: W, whole hemoglobin from the Sephadex G-25 column; F, M1, M2B, S, fractions from the DEAE column; F1, F2, F3, F4, fractions from preparative isoelectric focusing of fraction F. The anode is at the top of the gel.

fraction S from the DEAE column was further purified by preparative isoelectric focusing.

Isoelectric focusing in Sephadex gels on an analytical scale showed that fraction F could be resolved into four fractions in a number of different ampholyte mixtures. Preparative isoelectric focusing in a 3:1 mixture of pH 8-10.5 and pH 3-10 ampholytes provided a good separation of these four fractions (Fig. 12a), designated F1, F2, F3, and F4 based on their position in the focused gel, F1 being the closest to the cathode.

After removal from the gel and elution through Sephadex G-25 followed by dialysis to remove ampholytes, F1, F2, F3, and F4 each appeared to be pure on analytical (agarose) isoelectric focusing gels (Fig. 10) and on starch gels (Fig. 11).

None of the ampholyte systems tested in analytical isoelectric focusing in Sephadex gels was able to resolve fraction S from the DEAE column into distinct bands. Preparative isoelectric focusing of fraction S in a gel containing pH 5-8 ampholytes resulted in a broad band of hemoglobin about 4 cm wide (Fig. 12b). This band was divided into four sections which were then eluted through Sephadex G-25 and dialyzed to remove ampholytes. Analytical (agarose) isoelectric focusing showed small but detectable differences between these fractions (Fig. 13). The two fractions from each end of the broad band of hemoglobin were designated S1 and S2, with S1 being closest to the cathode. The two fractions from the middle of the band were not further characterized. The separation between S1 and S2 seen by analytical isoelectric focusing is also apparent on starch gel electrophoresis (Fig. 14) of these fractions.

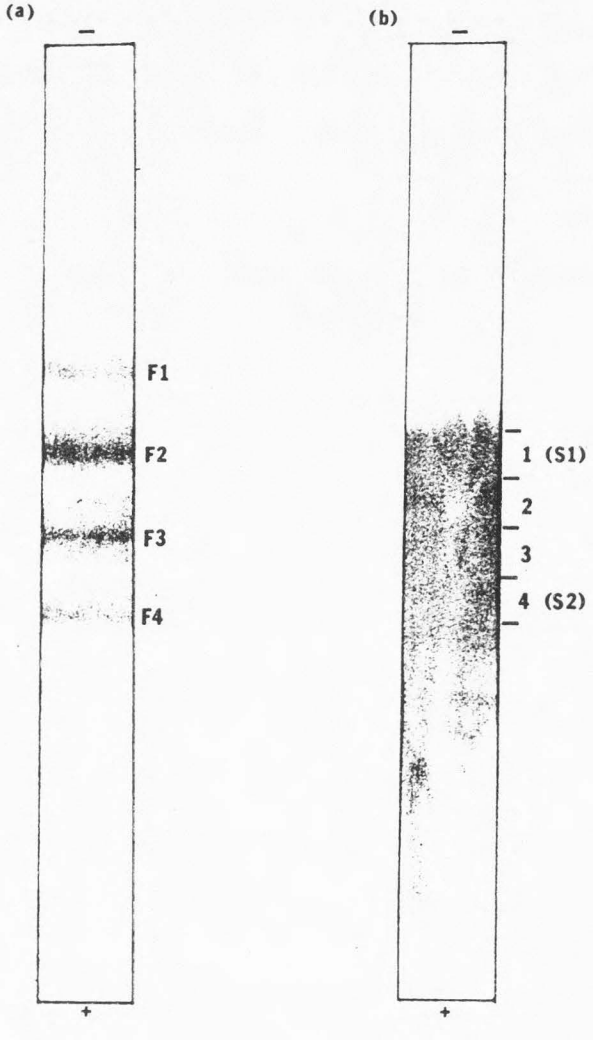


FIG. 12. **Preparative isoelectric focusing of partially purified cutthroat trout hemoglobins.** Paper prints of the gels after preparative isoelectric focusing of: (a) fraction F from the DEAE column; (b) fraction S from the DEAE column, with the fractions obtained from each gel indicated. Ampholytes used were: (a) pH 8-10.5 and pH 3-10, 3:1; (b) pH 5-8. The cathode is at the top of each print.

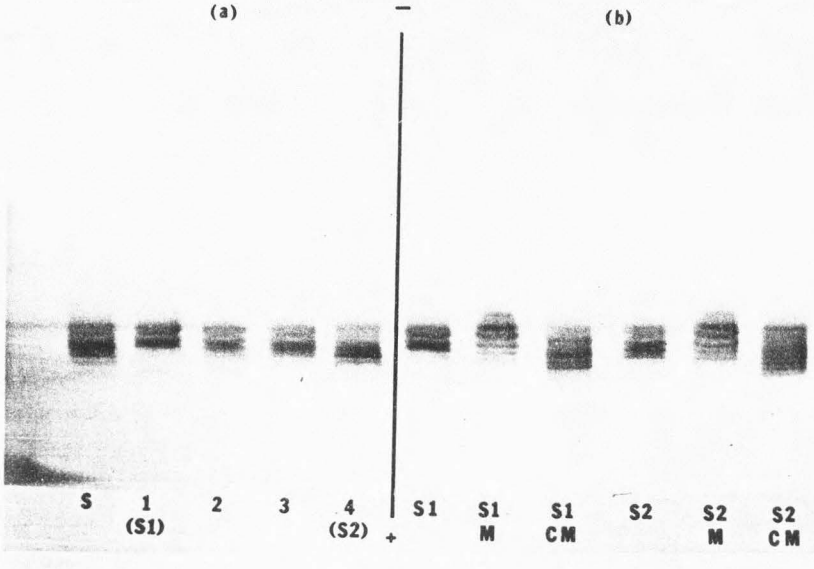


FIG. 13. **Analytical isoelectric focusing of cutthroat trout hemoglobins.** (a) Fractions applied are: S, fraction S from the DEAE column; 1, 2, 3, and 4, fractions from preparative IEF of fraction S (as labelled in Fig. 12b). Fractions 1 and 4 are designated S1 and S2 respectively. (b) Fractions S1 and S2 were applied to the gel as oxyhemoglobin (S1, S2), methemoglobin (S1M, S2M), and cyanomethemoglobin (S1CM, S2CM). Focusing conditions were as described in Materials and Methods except that a 1:1 mixture of pH 3-10 and pH 6.5-9 ampholytes was used. The cathode is at the top of the gel.

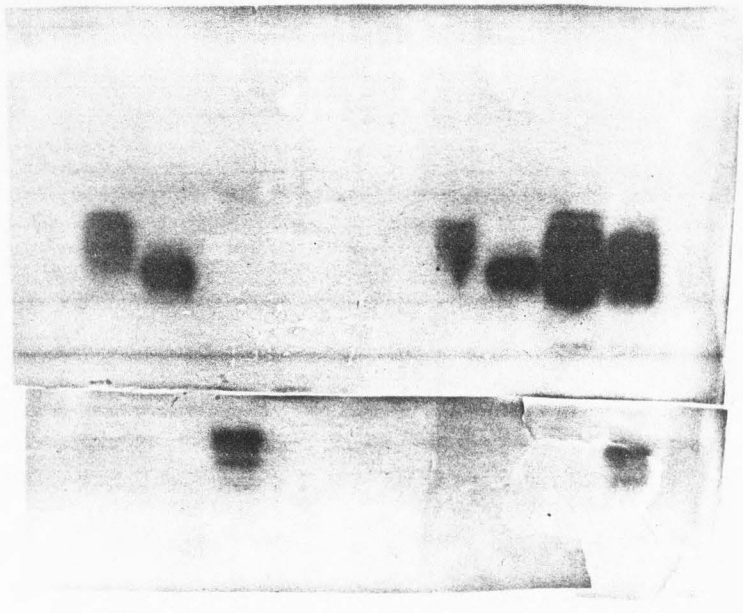


FIG. 14. **Starch gel electrophoresis of cutthroat trout hemoglobins.** Samples applied are: W, whole hemoglobin; F, M1, and S, fractions from the DEAE column; S1 and S2, fractions from preparative isoelectric focusing of fraction S. The gel was stained with o-dianisidine. The anode is at the top of the gel.

The results of a typical purification where fraction F from the DEAE column was subjected to preparative isoelectric focusing are summarized in Table III. Only one large scale separation of fraction S from the DEAE column was performed. In this case, 95mg of fraction S was applied to the preparative isoelectric focusing gel and 34mg of fraction S1 and 12mg of fraction S2 were obtained. The recovered fractions contained 4% and 6% methemoglobin, respectively.

By means of this purification procedure, nine distinct hemoglobin fractions were isolated from cutthroat trout. These fractions, M1, M2A, and M2B from the DEAE column and fractions F1, F2, F3, F4, S1, and S2 from preparative isoelectric focusing gels, are designated Hb M1, Hb M2A, Hb M2B, Hb F1, Hb F2, Hb F3, Hb F4, Hb S1, and Hb S2 respectively. The partially purified fractions from the DEAE column are likewise designated Hb F and Hb S.

Spectral properties of cutthroat trout hemoglobins—The UV-visible spectral properties of cutthroat trout hemoglobins were studied primarily to determine the validity of using the extinction coefficients for human hemoglobin to calculate the concentration of oxy-, deoxy-, and methemoglobin for these hemoglobins.

A sample of the whole hemoglobin was diluted in 5 mM Tris, 0.1 M NaCl, pH 8.2 to give an absorbance at 576 nm of approximately 0.3 and the spectrum from 650 nm to 500 nm was measured (Fig. 15). This solution was then diluted 1:11 in the same buffer and the spectrum from 500 nm to 250 nm was recorded. The total heme concentration calculated from the absorbance at 560, 576, and 630 nm (20.6 uM, 97% oxyhemoglobin) was used to calculate the millimolar extinction coef-

TABLE III

Results for a typical purification of cutthroat trout hemoglobins

Blood (12ml) from three fish was pooled and the hemoglobins purified as described in Materials and Methods. Data for preparative IEF fractions are for the fractions after G-25 chromatography and dialysis to remove ampholytes.

Purification step	[Hb] mg/ml	volume (ml)	mg Hb	successive yield (%)	% metHb
Crude hemolyzate	31	17	530	(100)	0
Sephadex G-25 chromatography	14	35	490	92	0
Dialysis	13	36	470	96	0.7
DEAE cellulose chromatography (fractions)					
F	3.1	36	110		1.0
M1	4.0	5.9	24		0
M2A	0.55	1.2	0.66		2.0
M2B	1.2	2.9	3.5		0.5
S	25	9.6	240		1.0
		total:	380	80	
Concentration of fraction F	8.7	12	100	90	2.6
Preparative isoelectric focusing (fractions)					
F1	4.2	3.7	16		3.2
F2	8.1	4.9	40		3.0
F3	5.2	3.8	20		2.7
F4	3.1	3.8	12		3.3
		total:	88	88	

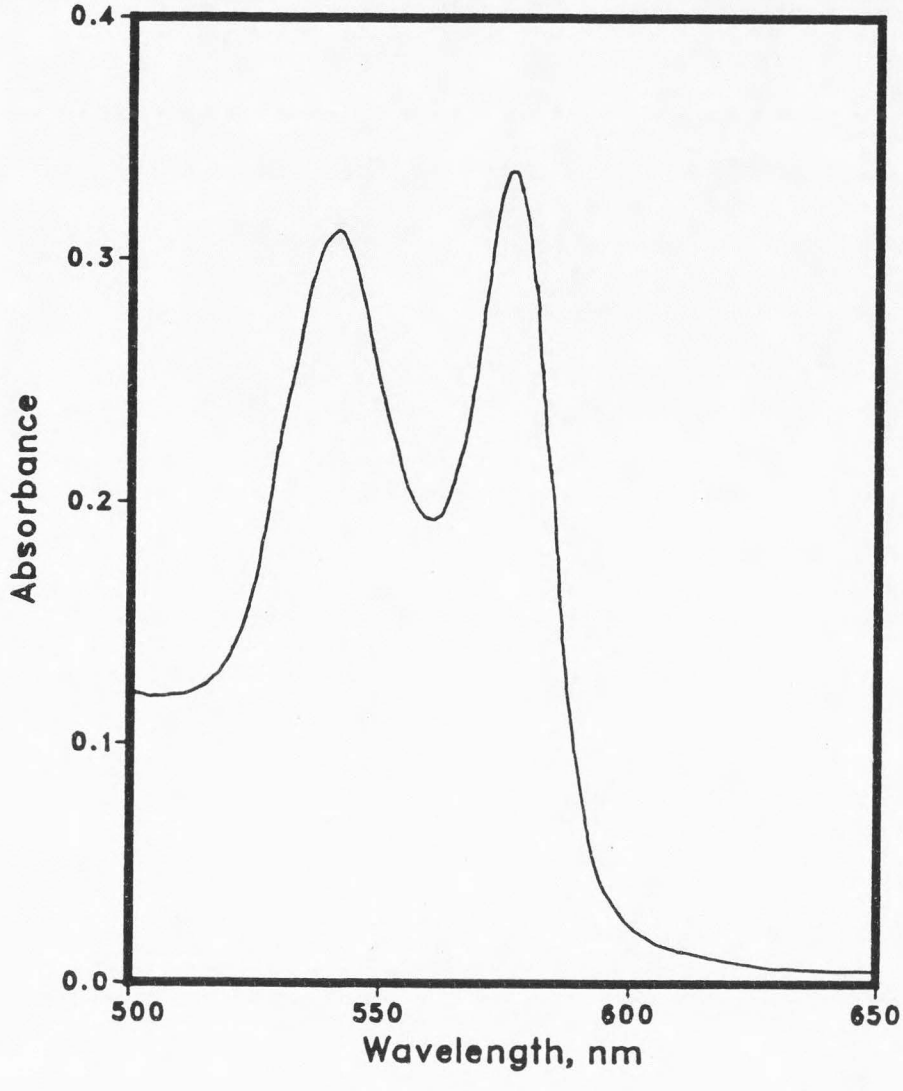


FIG. 15. **Visible absorption spectrum of cutthroat trout whole hemoglobin.** Whole hemoglobin in 5 mM Tris, 0.1 M NaCl, pH 8.2 at 20°C.

ficients for the 500-250 nm spectrum (Fig. 16). The wavelengths and the calculated extinction coefficients of the three absorption maxima in this region are compared to the values for human hemoglobin in Table IV.

To determine whether the absorption spectrum of cutthroat trout methemoglobin shows a pH dependence similar to human methemoglobin, a sample of whole hemoglobin was oxidized to methemoglobin as described for analytical isoelectric focusing in Materials and Methods. Aliquots of the oxidized hemoglobin were dialyzed against 50 mM Taps, 0.1 M NaCl, pH 9.0, and 50 mM Bis-tris, 0.1 M NaCl, pH 6.2 and the spectra from 650 nm to 480 nm recorded. The two spectra (Fig. 17) are essentially identical to the spectra for human methemoglobin at pH 6.5 and pH 9.0 (133).

In addition to the spectral data described above for the whole hemoglobin from cutthroat trout, the absorption spectra in the 650-500 nm region were measured for some of the purified hemoglobins in the oxygenated and deoxygenated states. These spectra were recorded in 50 mM Tes, 0.1 M NaCl, pH 7.4 at 20°C. Samples were deoxygenated as described for oxygen equilibrium measurements. The spectra for Hb F1 are shown in Fig. 18. All the observed spectra are similar to those for human oxy- and deoxyhemoglobin. Table V shows the observed absorption maxima for cutthroat trout hemoglobins in the 500-650 nm region.

Isoelectric points of cutthroat trout hemoglobins—The isoelectric points of cutthroat trout hemoglobins were determined by isoelectric focusing in agarose gels. The whole hemoglobin,

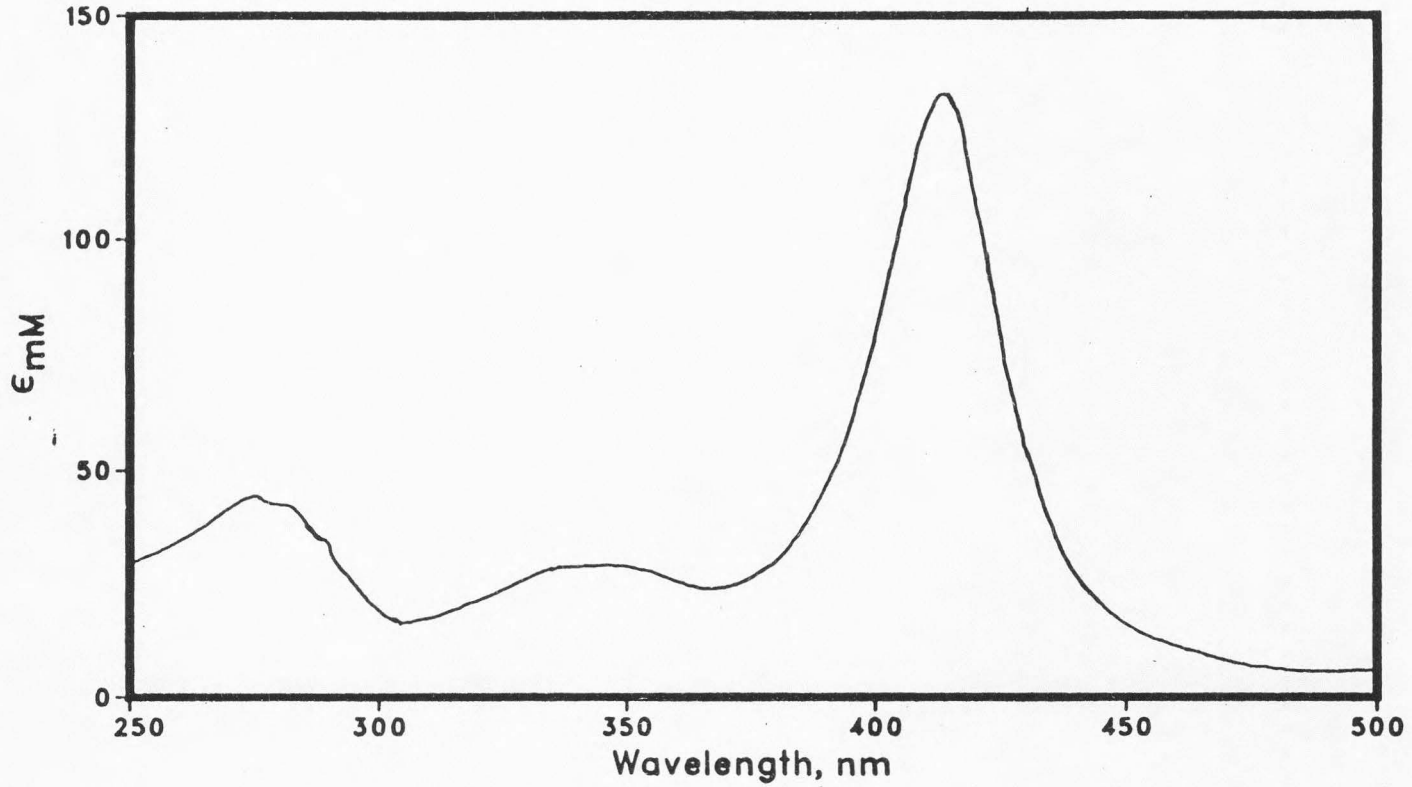


FIG. 16. **UV-visible absorption spectrum of cutthroat trout whole hemoglobin.** Whole hemoglobin (1.9 uM) in 5 mM Tris, 0.1 M NaCl, pH 8.2 at 20°C. Extinction coefficients calculated as described in the text.

TABLE IV

UV-visible spectral data for cutthroat trout whole hemoglobin

Data for cutthroat trout hemoglobin from spectra at pH 8.2 and 20°C (see text for details), for human hemoglobin from spectra at pH 7, 20°C (from reference 133).

	λ_{max}	ϵ_{mM}	λ_{max}	ϵ_{mM}	λ_{max}	ϵ_{mM}
Cutthroat trout whole oxyhemoglobin	414	130	345	28	276	43
Human oxyhemoglobin	415	125	344	27	276	34.4

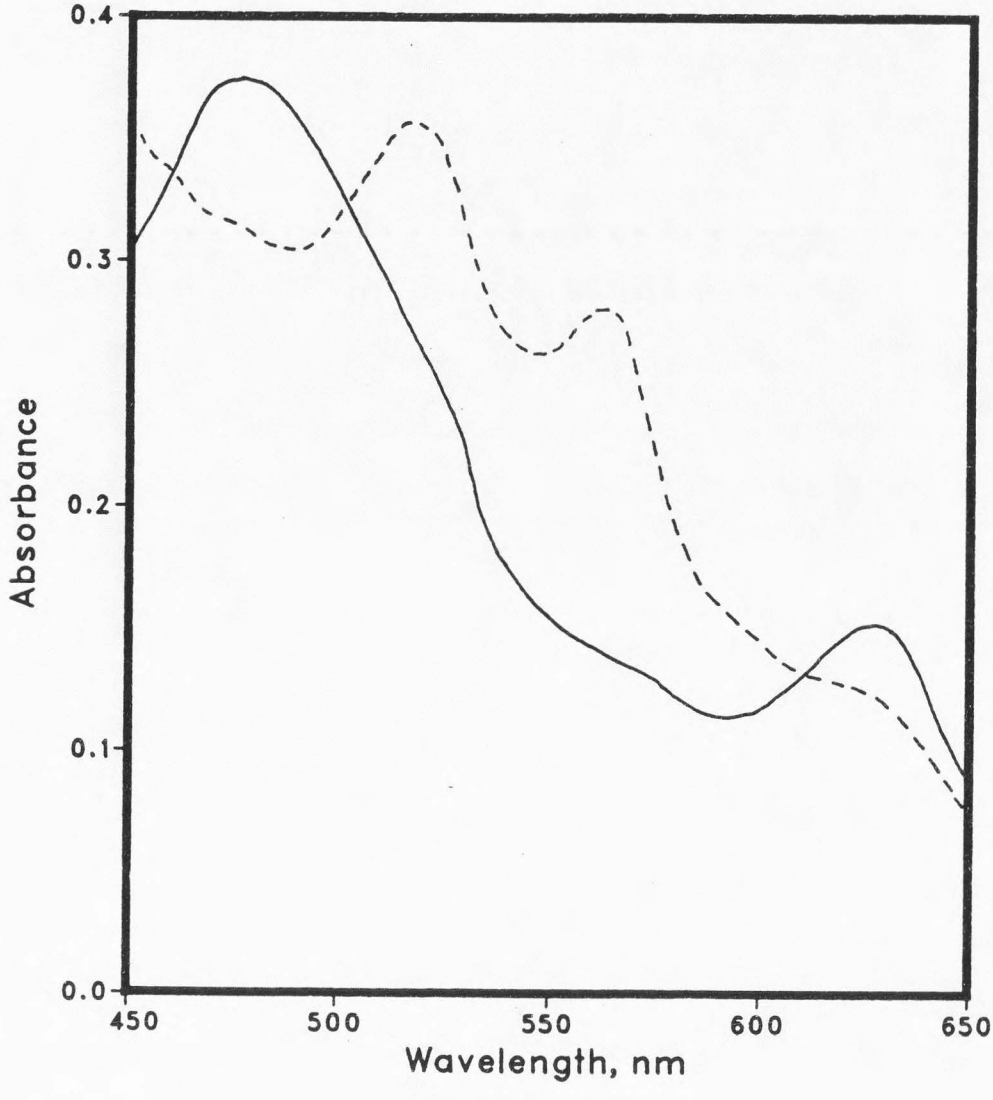


FIG. 17. **Visible absorption spectra of cutthroat trout whole methemoglobin.** Ferricyanide oxidized whole hemoglobin at pH 6.2 (———), and at pH 9.0 (-----).

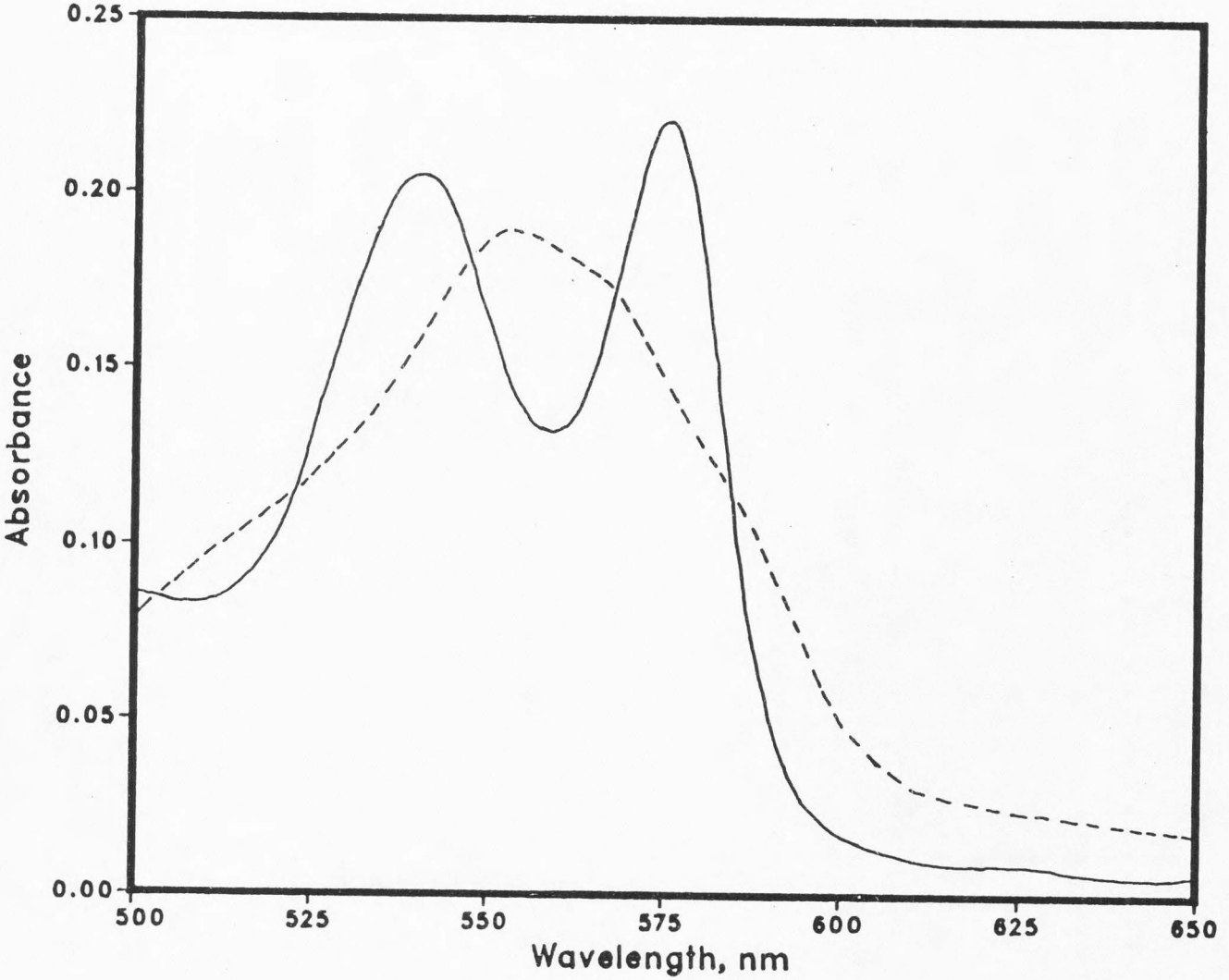


FIG. 18. **Visible absorption spectra of Hb Fl.** Spectra in 50 mM Tes, 0.1 M NaCl, pH 7.4 at 20°C. (-----), oxyHb Fl; (-----), deoxyHb Fl.

TABLE V

Absorption maxima of cutthroat trout hemoglobins in the 500-650 nm region

Spectra for purified cutthroat trout hemoglobins at pH 7.4, for whole oxyhemoglobin at pH 8.2, for human oxy- and deoxyhemoglobin at pH 7. All values for human hemoglobin from reference 133.

	λ_{max}, nm						
	oxyHb		deoxyHb	acidic[a] metHb		alkaline[b] metHb	
Cutthroat trout Hb:							
Whole	541	576		502	632	538	575
F1	540	575	554				
M1	540	575	553				
M2A	541	576	553				
M2B	540	576	554				
S1	540	576	554				
Human Hb	541	576	555	500	631	540	575

[a]At pH 6.2 for cutthroat trout Hb, pH 6.5 for human Hb.

[b]At pH 9.0.

fractions F and S from the DEAE column, and the purified hemoglobins; Hb F1, Hb F2, Hb F3, Hb F4, Hb M1, and Hb M2B were applied to the gel along with the pI marker proteins. The stained gel is shown in Fig. 19. In this gel, Hb S focused as two distinct bands which were assumed to represent Hb S1 and Hb S2. The pH gradient for this gel is shown in Fig. 20, with the positions of the focused hemoglobins indicated. The isoelectric points of the hemoglobins determined from this pH gradient are given in Table VI.

Molecular weight of cutthroat trout hemoglobin—The molecular weight of cutthroat trout whole hemoglobin was determined by gel exclusion chromatography on Sephadex G-100. The column was calibrated by chromatography of proteins of known molecular weight (Fig. 21). A relatively large amount (15 mg) of hemoglobin was applied to the column in order to detect components of different molecular weight which might be present in low proportions, e.g. Hb M2A and Hb M2B. The hemoglobin eluted from the column as a single symetrical peak with an elution volume corresponding to a molecular weight of 64,000 (Fig. 22), indicating that the cutthroat trout hemoglobins all have the tetrameric structure typical of vertebrate hemoglobins.

Oxygen equilibria of cutthroat trout hemoglobins—In order to determine the reproducibility of oxygen equilibrium measurements, several oxygen equilibrium curves were determined in triplicate. the results of three measurements of the curve for Hb F1 at pH 7.1 and 20°C are shown in Fig. 23. In general, the values of Y calculated for each run agree well in the 10% to 90% saturation range (log (Y/1-Y) = -1.0 to +1.0), with greater deviations apparent at either

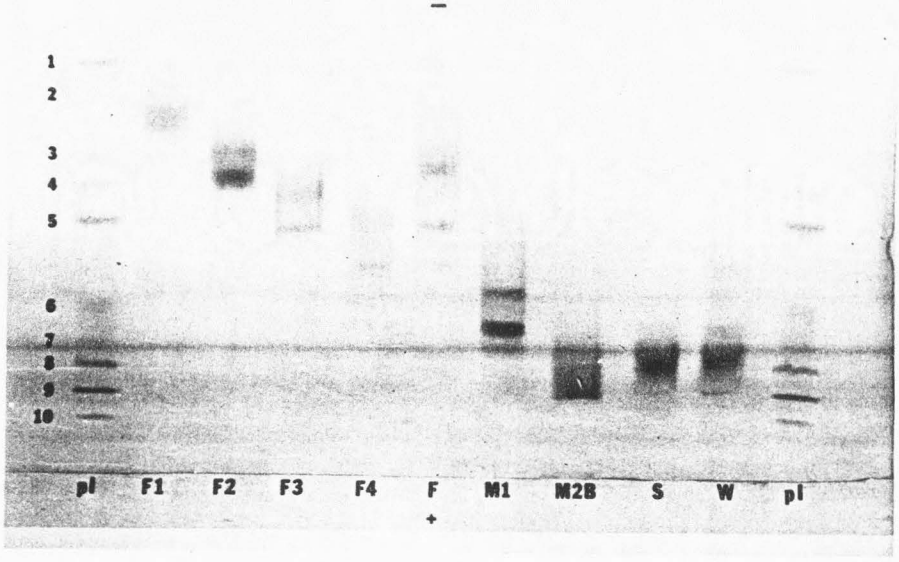


FIG. 19. **Analytical isoelectric focusing for determination of isoelectric points.** Samples are cutthroat trout hemoglobins and marker proteins (pI) as indicated. The marker proteins and their isoelectric points are: 1, cytochrome C, 10.25; 2, trypsinogen, 9.30; 3, lentil lectin-basic band, 8.65; 4, lentil lectin-middle band, 8.45; 5, lentil lectin-acidic band, 8.15; 6, horse myoglobin-basic band, 7.35; 7, horse myoglobin-acidic band, 6.85; 8, human carbonic anhydrase B, 6.55; 9, bovine carbonic anhydrase B, 5.85; 10, B-lactoglobulin A, 5.20. Focusing conditions as described in Materials and Methods. The cathode is at the top of the gel.

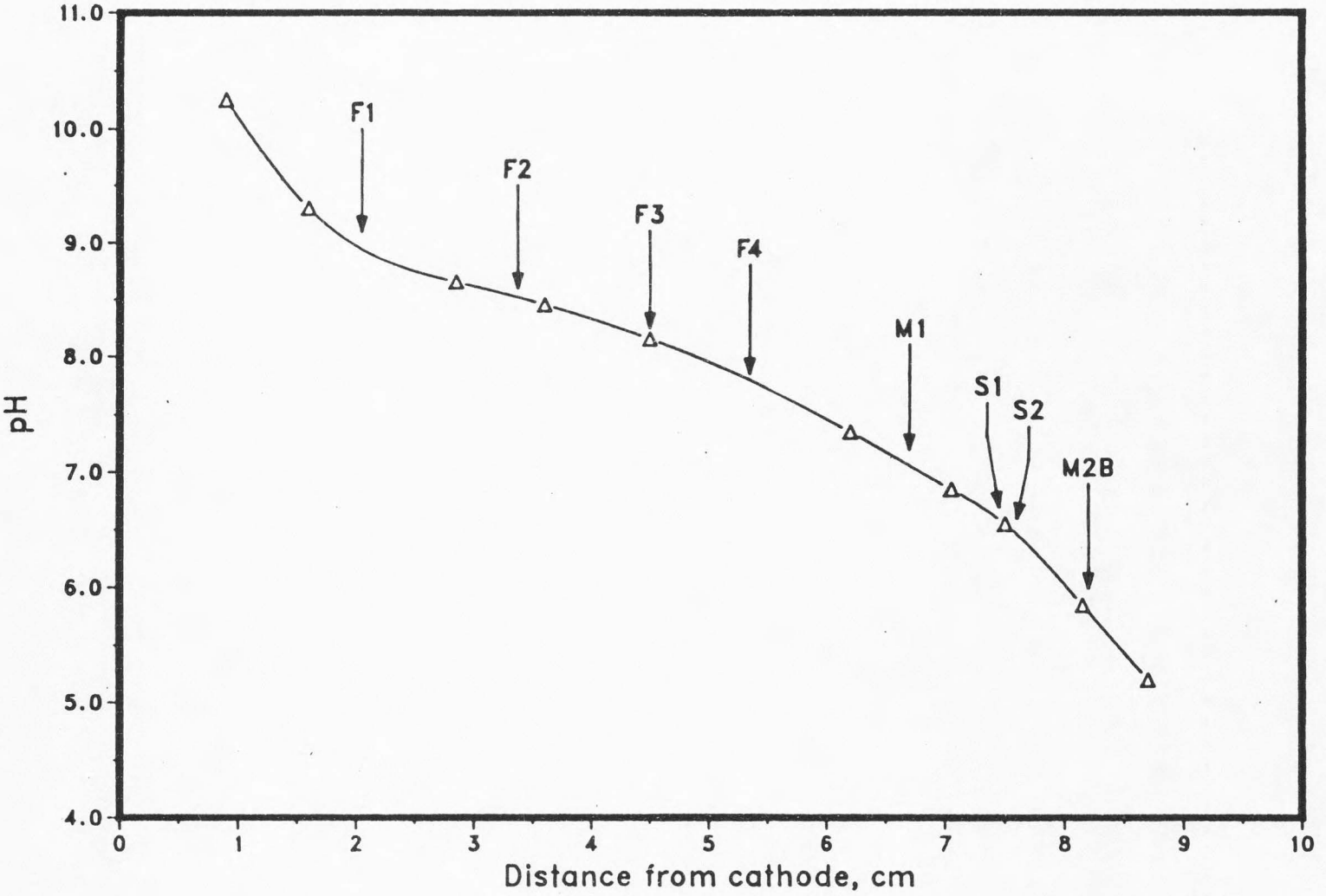


FIG. 20. **pH gradient for determination of isoelectric points.** The pH gradient was constructed from the positions of the marker proteins in the gel shown in Fig. 19. Positions of the hemoglobins are indicated by the arrows. Distances were measured to the center of each band.

TABLE VI

Isoelectric points of cutthroat trout hemoglobins

Isoelectric points determined by isoelectric focusing in agarose and comparison of positions to marker proteins. For hemoglobins focusing as diffuse bands a range is given. Other values were calculated from the distance to the center of each band. The temperature in the gel was not directly measured. According to Pharmacia (118) the temperature is 24 +/- 1.5°C under these conditions.

Hemoglobin	Isoelectric point
F1	8.90-9.15
F2	8.45-8.55
F3	8.15
F4	7.75
M1	7.00-7.15
S1	6.55
S2	6.45
M2B	5.90

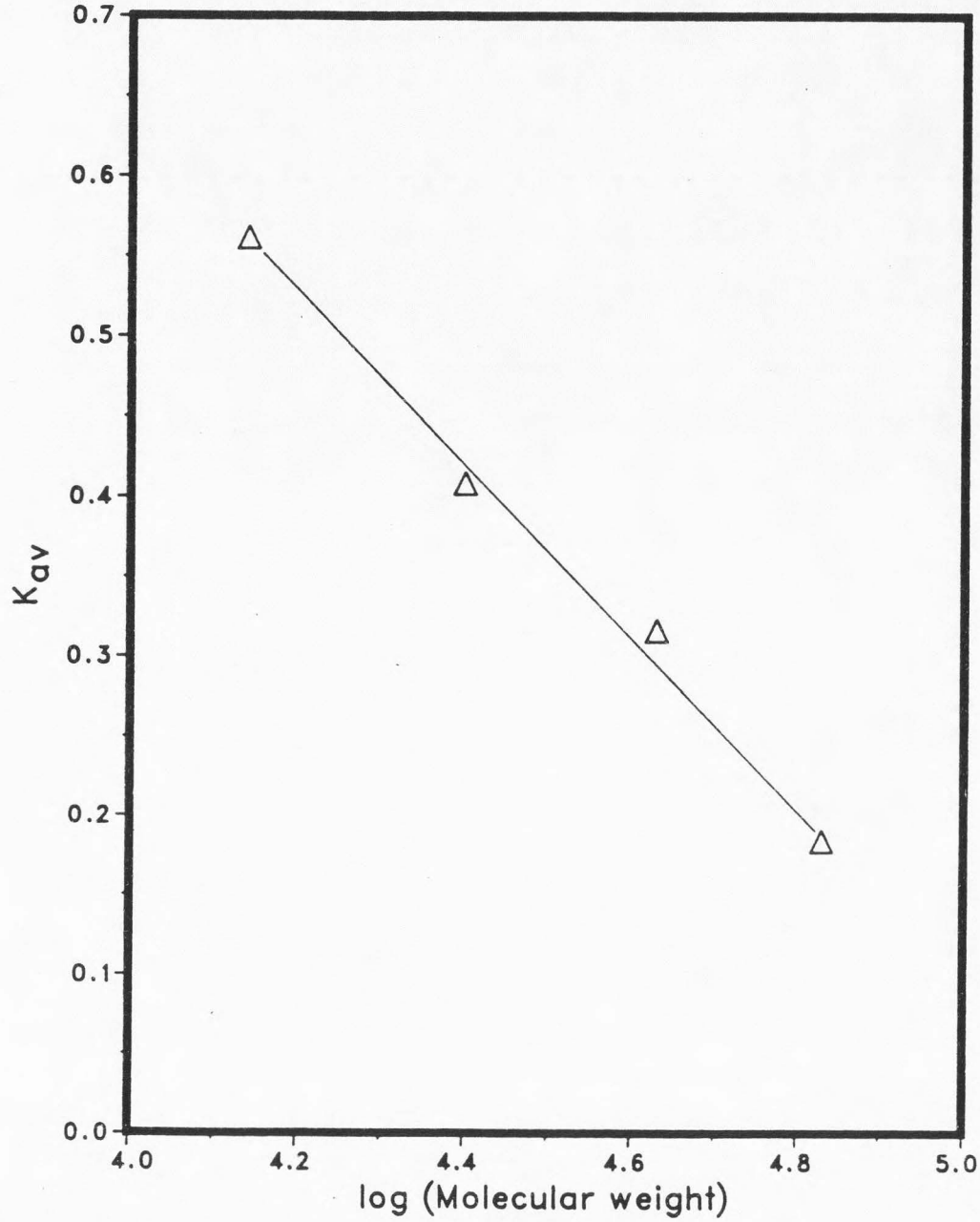


FIG. 21. **Calibration curve for determination of molecular weight.** Molecular weight standards are: ribonuclease, 13,700; chymotrypsinogen, 25,000; ovalbumin, 43,000; and albumin, 67,000.

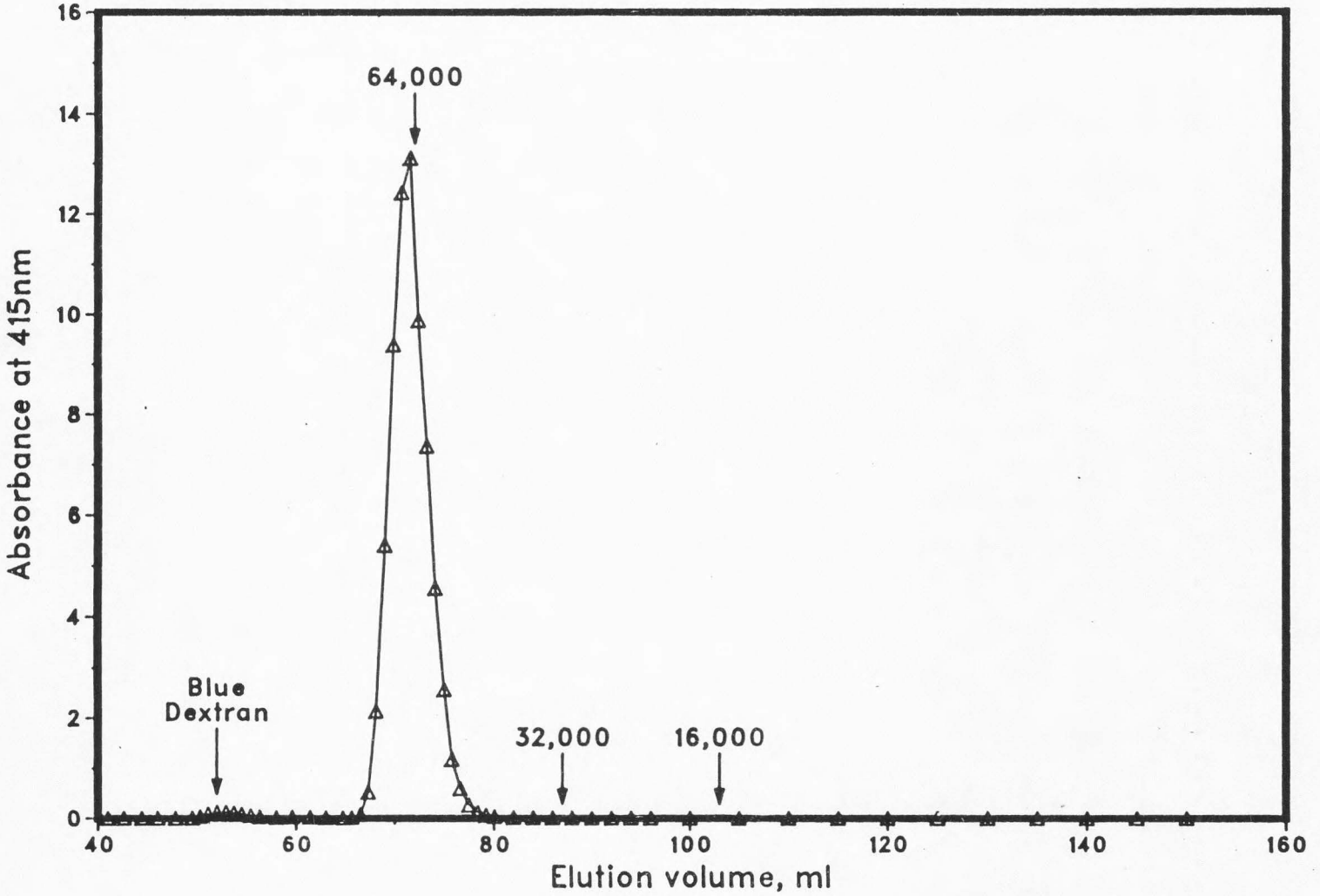


FIG. 22. **Chromatography of cutthroat trout whole hemoglobin on Sephadex G-100.** Whole Hb (15 mg) was applied to the column and eluted with 5 mM Tris, 0.1 M NaCl, pH 8.2. Molecular weights were calculated from the calibration curve (Fig. 21).

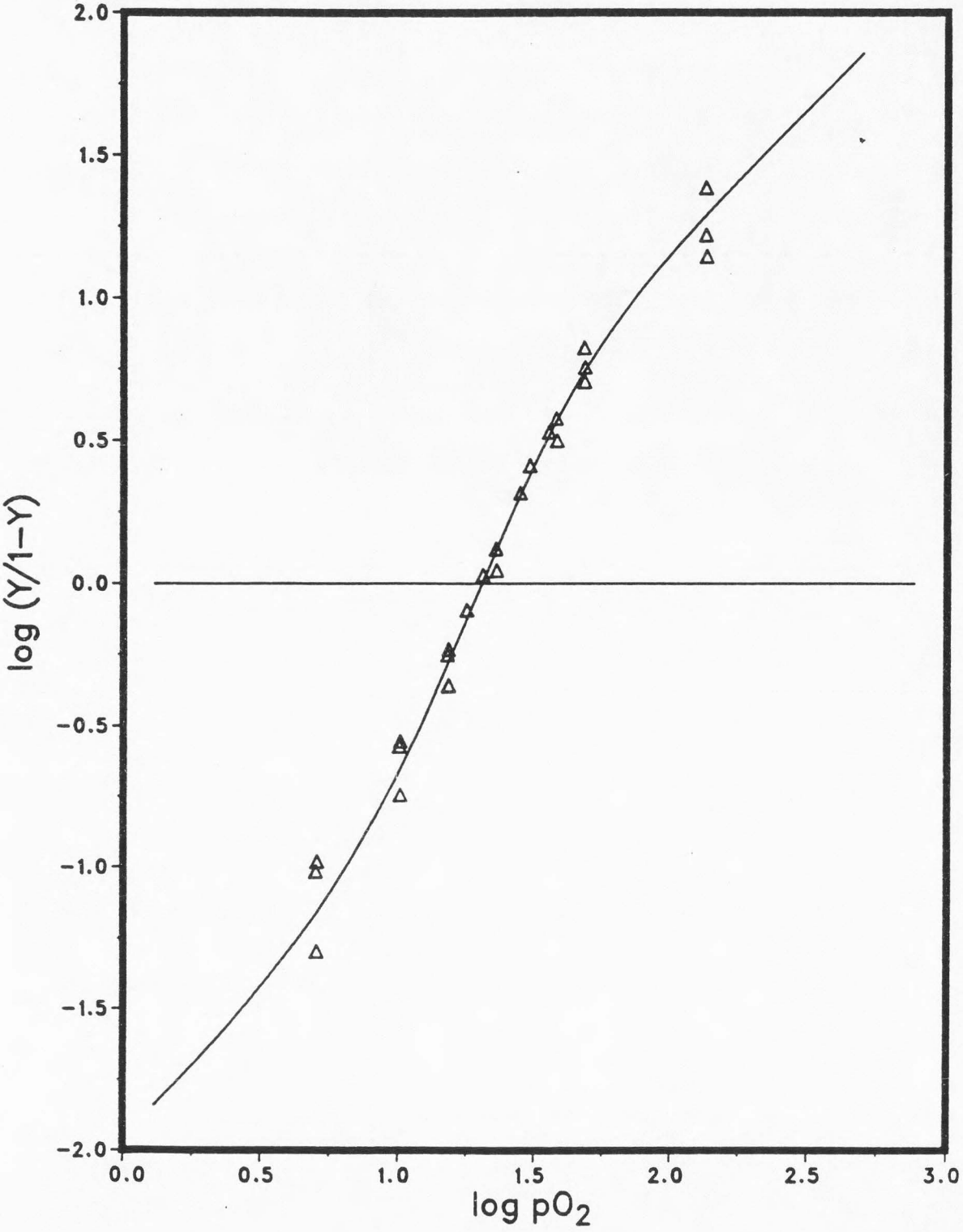


FIG. 23. **Reproducibility of oxygen equilibrium measurements.** The data for three determinations of the oxygen equilibrium curve of Hb F1 at pH 7.1 and 20°C are shown. The line represents the theoretical curve fitted to all the data. Conditions as described in Materials and Methods.

extreme of the curve. The errors in the values of $n_{1/2}$ and log $p_{1/2}$ determined from the oxygen equilibrium curves were estimated from the standard deviations of $n_{1/2}$ and log $p_{1/2}$ for the four curves which were measured in triplicate (Hb F1, Hb F2, and Hb M2B at pH 7.1, and Hb M2B at pH 8.0, all at 20°C). The average of the standard deviations for these four curves were 0.07 for $n_{1/2}$ and 0.03 for log $p_{1/2}$. Based on these results, we estimate the errors in these parameters to be +/- 0.1 for $n_{1/2}$ and +/- 0.05 for log $p_{1/2}$. Except for this estimation of errors, where more than one determination on a given curve was made, the data were combined and fitted to a single theoretical curve for calculation of $n_{1/2}$ and log $p_{1/2}$.

In most of these measurements, methemoglobin was present at about 5% initially and increased by 2-5% during the determination of an oxygen equilibrium curve. Formation of methemoglobin was more pronounced at low pH (pH 6.2 and pH 6.5) and at high temperatures (25°C and 30°C), but never increased by more than 10% in any run. The final percentage of methemoglobin never exceeded 20%.

Three determinations of the oxygen equilibrium curve of Hb M2B (at pH 7.1 and 20°C) in the presence of increasing concentrations of methemoglobin are shown in Fig. 24. These measurements were made 4 (0-2% metHb), 7 (3-6% metHb), and 11 (12-18% metHb) days after obtaining blood. The increase in the initial percentage of methemoglobin present (0, 3, and 12%) reflects the rate of oxidation of the heme iron during storage at 4°C and pH 7.7. The percentage of methemoglobin present at the end of each run (2, 6, and 18%) shows that oxidation during the measurements is more rapid as the initial concentration of methemoglobin increases. Although there is a slight

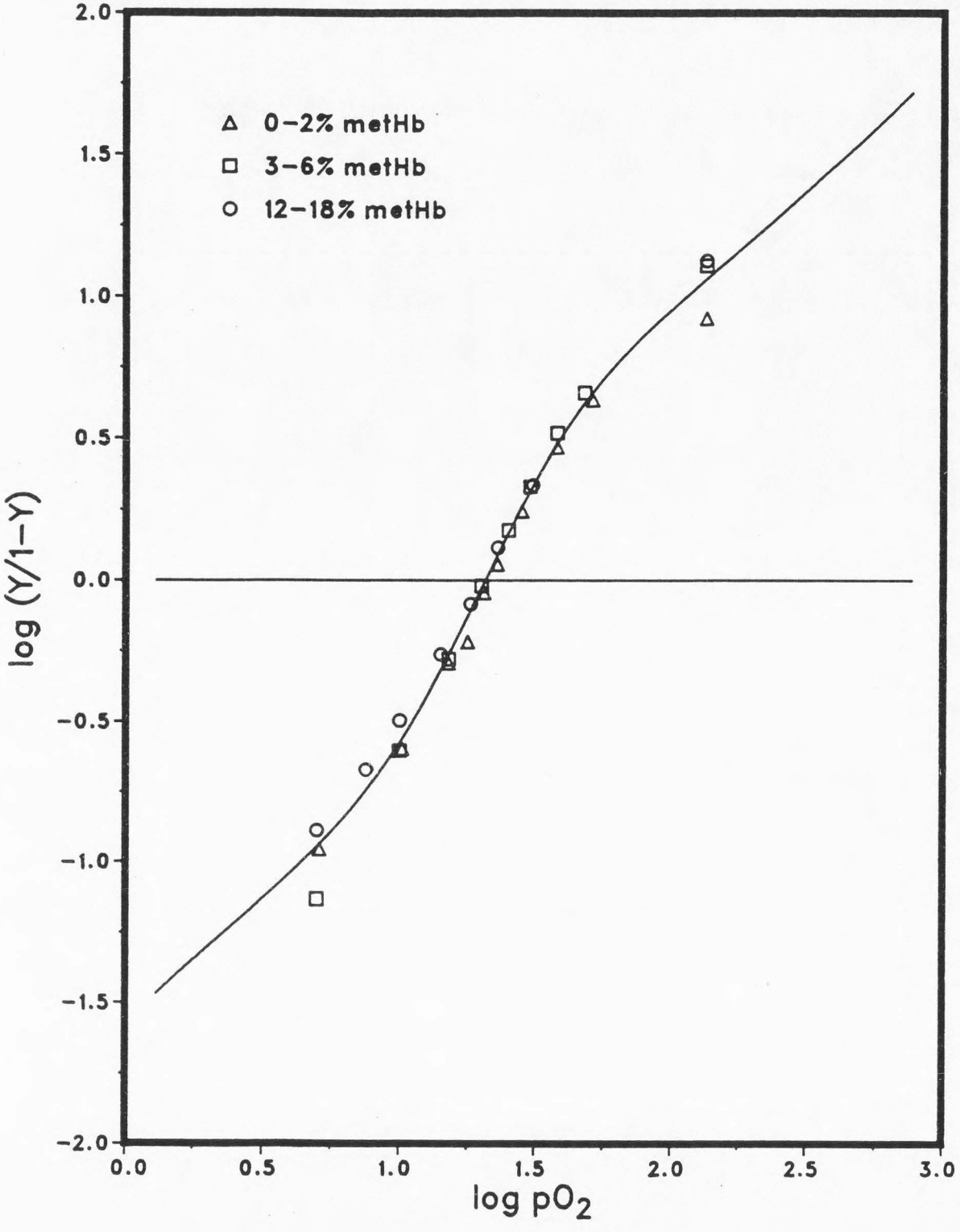


FIG. 24. **Effect of methemoglobin on oxygen eqiulibrium measurements.** The data for three determinations of the oxygen equilibrium curve of Hb M2B at pH 7.1 and 20°C are shown. The percentages of methemoglobin given are the initial and final percentages for each run. The curves were determined 4 (0-2% metHb), 7 (3-6% metHb), and 11 (12-18% metHb) days after blood was obtained.

trend towards higher oxygen affinity at the higher methemoglobin concentrations, the differences in the curves are within experimental error, as can be seen by a comparison of Figs. 23 and 24 (methemoglobin concentrations were < 5% for the three determinations in Fig. 23).

The pH dependence of the oxygen binding equilibria of the purified hemoglobins was studied from pH 6.2 to pH 8.0 at 20°C. Within this range, the oxygen equilibrium curves of all the hemoglobins except Hb M2A, Hb S1, and Hb S2 display very little change with pH. This is illustrated for Hb F3 in Fig. 25. The Hill plots for Hb M2A at pH 8.0, pH 7.1, and pH 6.2 (Fig. 26) show that the oxygen equilibrium of this hemoglobin is pH dependent, with the curves shifted to lower oxygen pressures at pH 8.0 and pH 6.2. In Fig. 27, it can be seen that the oxygen equilibrium curve of Hb S2 is strongly pH dependent, with the curve shifted to higher oxygen pressures as the pH is decreased. Very similar results were obtained with Hb S1. The values of $n_{1/2}$ and log $p_{1/2}$ obtained from the equilibrium curves for each hemoglobin as a function of pH are shown in Figs. 28, 29, and 30.

The effect of ATP and GTP on the oxygen equilibria of the purified hemoglobins was observed by comparison of the equilibrium curve for each of the purified hemoglobins at pH 7.1 and 20°C without added nucleotides and in the presence of saturating levels of ATP or GTP. ATP had no effect on the oxygen equilibria of the cathodal hemoglobins or Hb M1, Hb M2A, and Hb M2B. This is shown for Hb F3 in Fig. 31. In contrast, the equilibrium curves for Hb S1 and Hb S2 are shifted to much higher oxygen pressures in the presence of

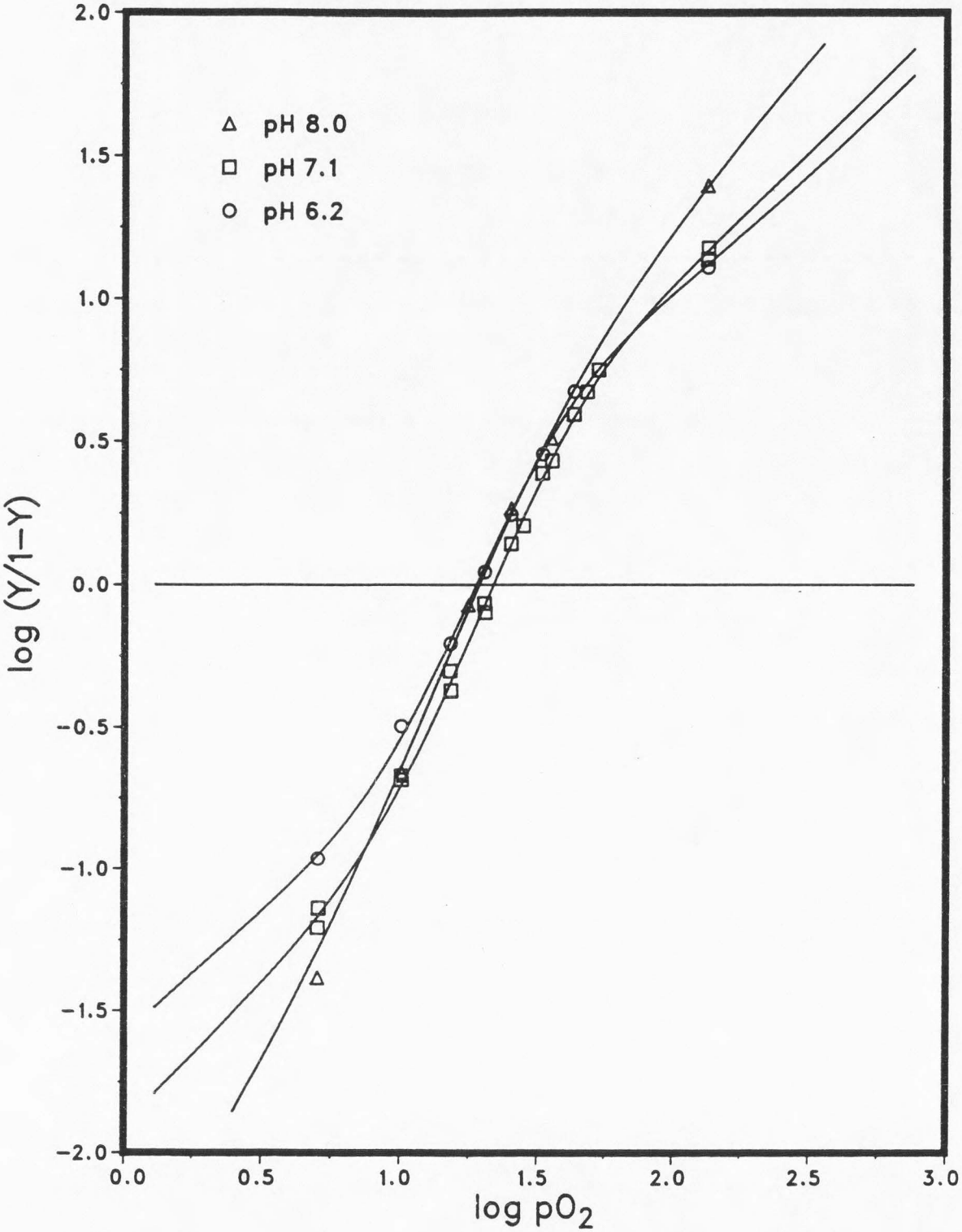


FIG. 25. **pH dependence of oxygen equilibrium of Hb F3.** Oxygen equilibrium curves at pH 8.0, 7.1, and 6.2 (20°C) as indicated. Conditions as described in Materials and Methods.

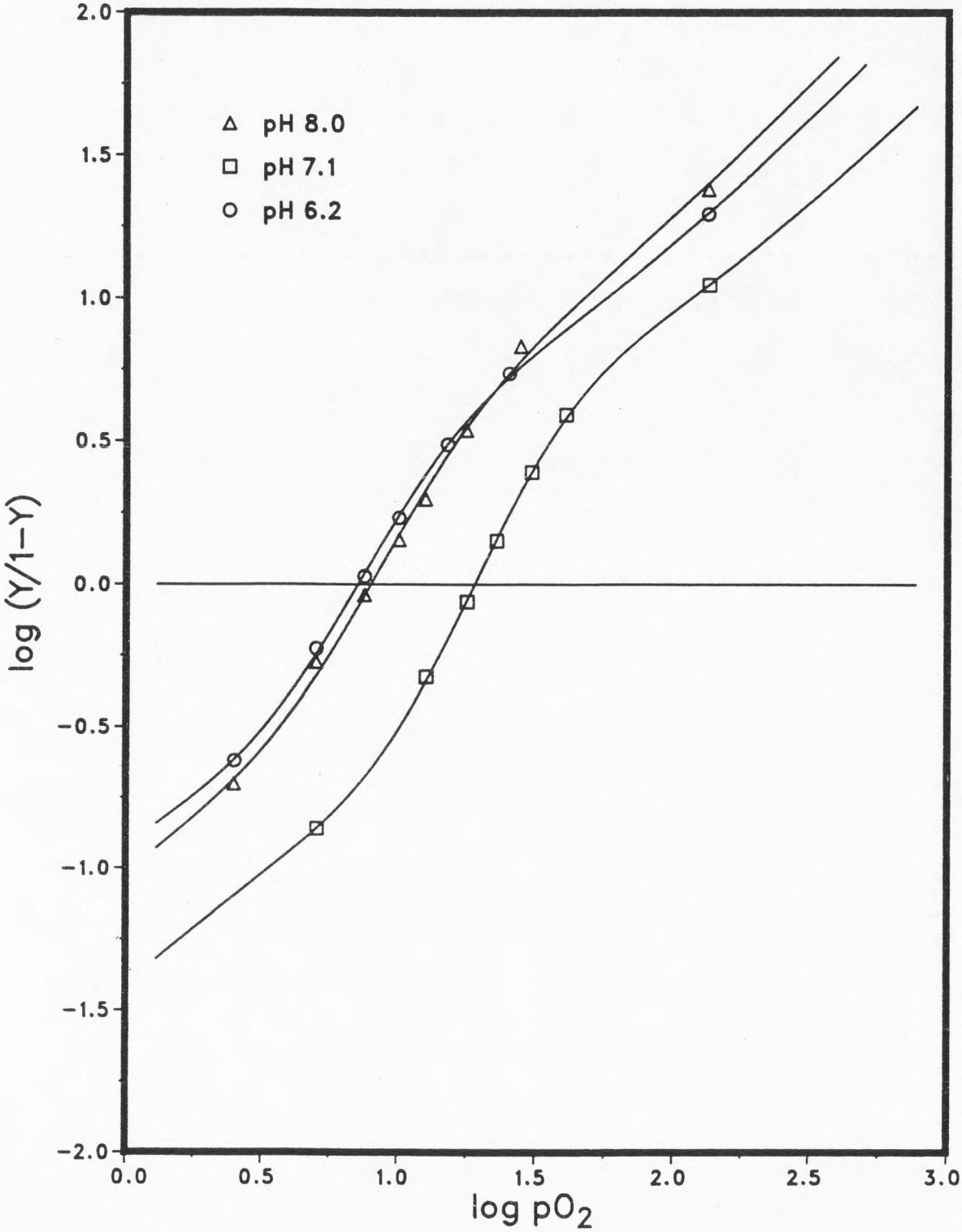


FIG. 26. **pH dependence of oxygen equilibrium of Hb M2A.** Oxygen equilibrium curves at pH 8.0, 7.1, and 6.2 ($20^{o}C$) as indicated. Conditions as described in Materials and Methods.

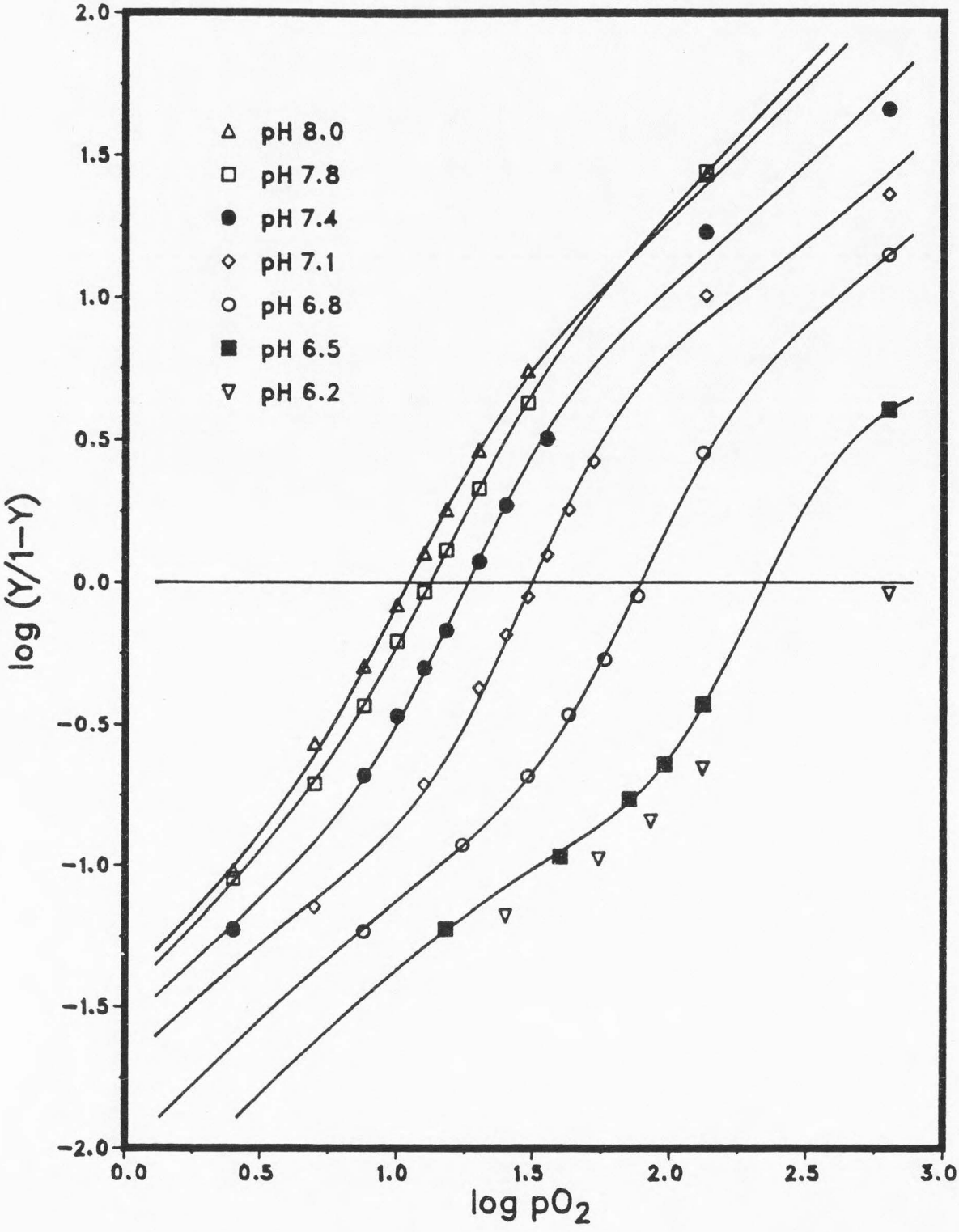


FIG. 27. **pH dependence of oxygen equilibrium of Hb S2.** Oxygen equilibrium curves at pH 8.0, 7.8, 7.4, 7.1, 6.8, 6.5, and 6.2 (20°C) as indicated. A theoretical curve could not be fitted to the data at pH 6.2. Conditions as described in Materials and Methods.

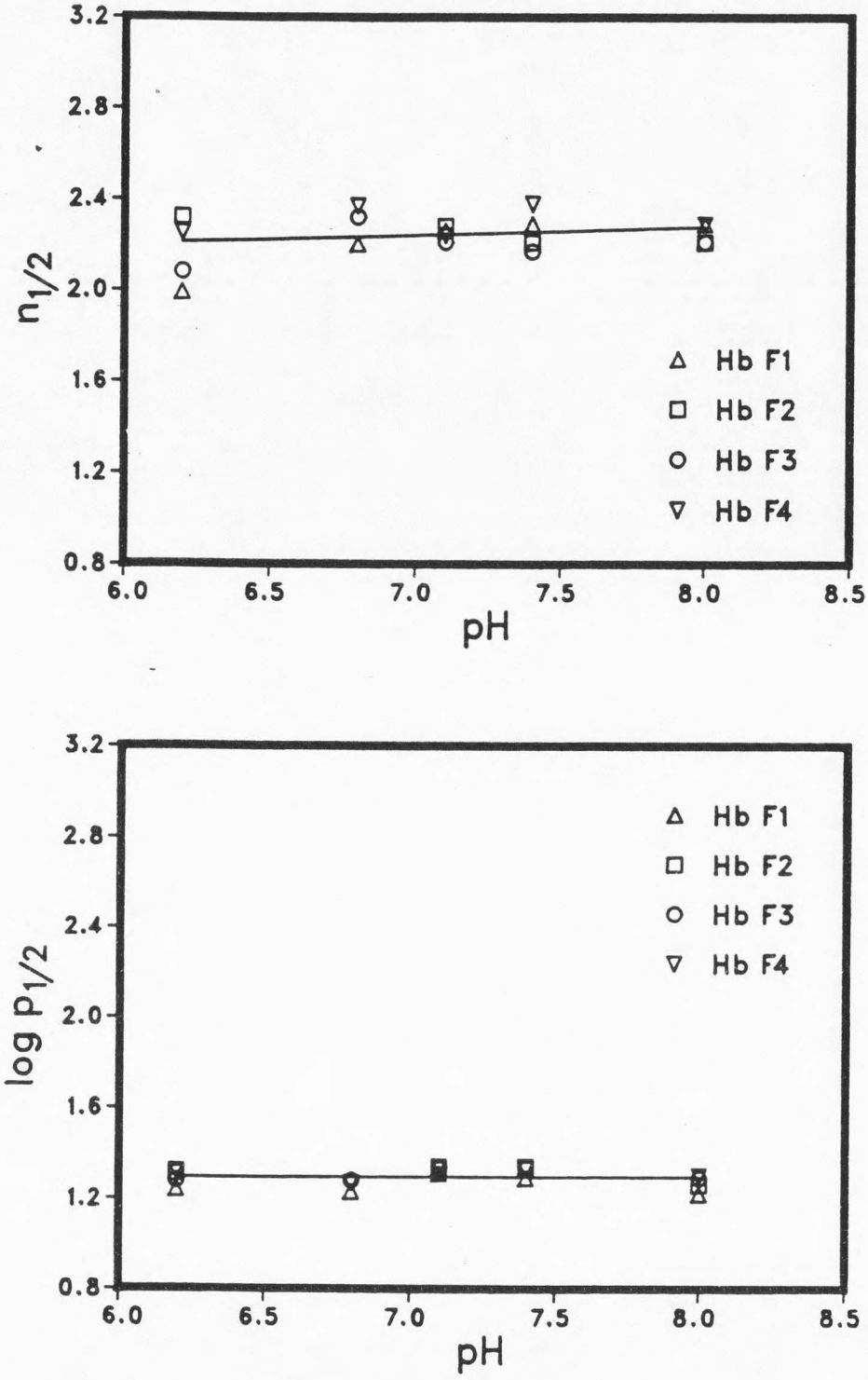


FIG. 28. **pH dependence of oxygen equilibrium parameters of Hb F1, Hb F2, Hb F3, and Hb F4.** The values of $n_{1/2}$ and log $p_{1/2}$ from the equilibrium curves for each hemoglobin as indicated.

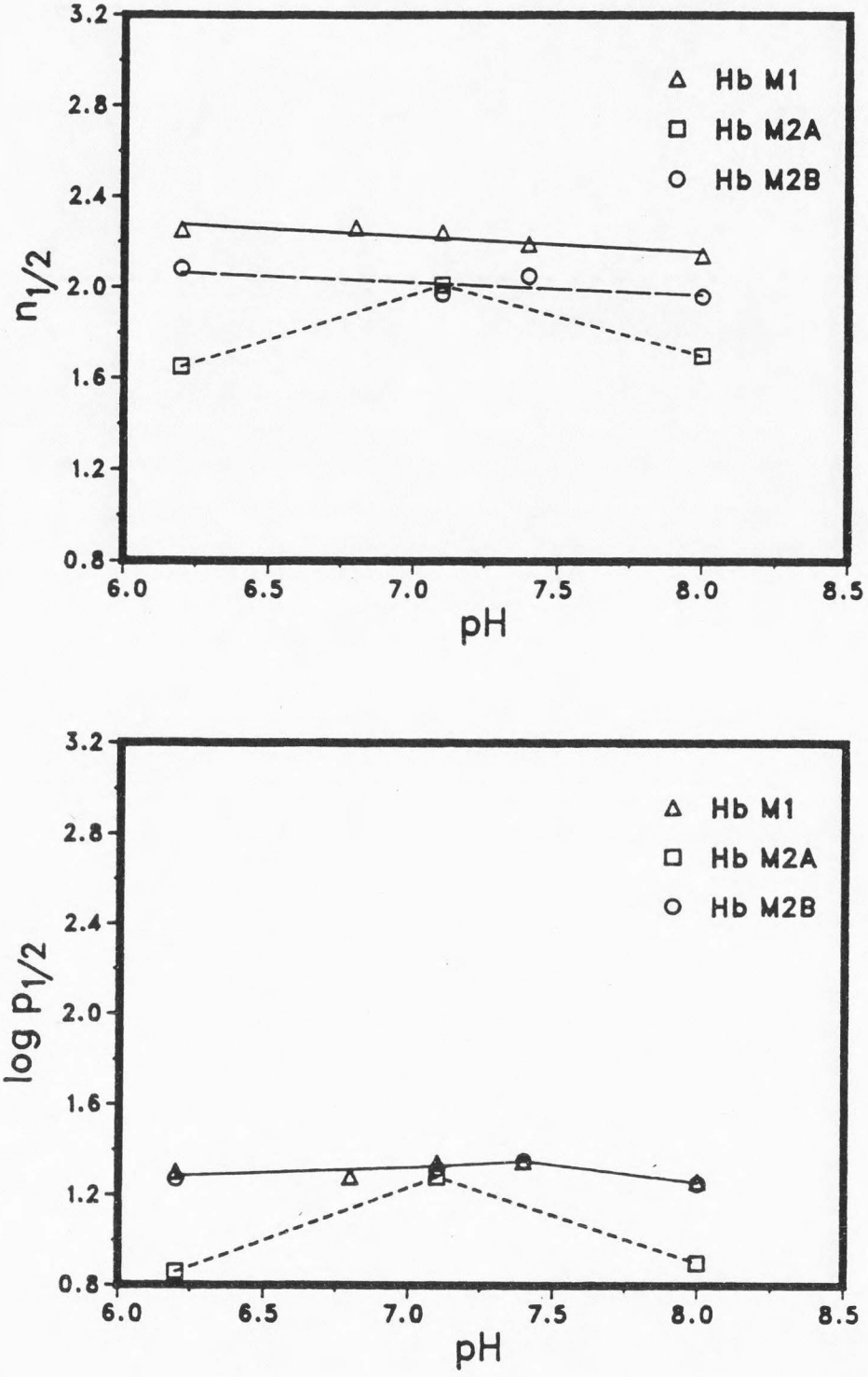


FIG. 29. **pH dependence of oxygen equilibrium parameters of Hb M1, Hb M2A, and Hb M2B.** The values of $n_{1/2}$ and log $p_{1/2}$ from the equilibrium curves for each hemoglobin as indicated.

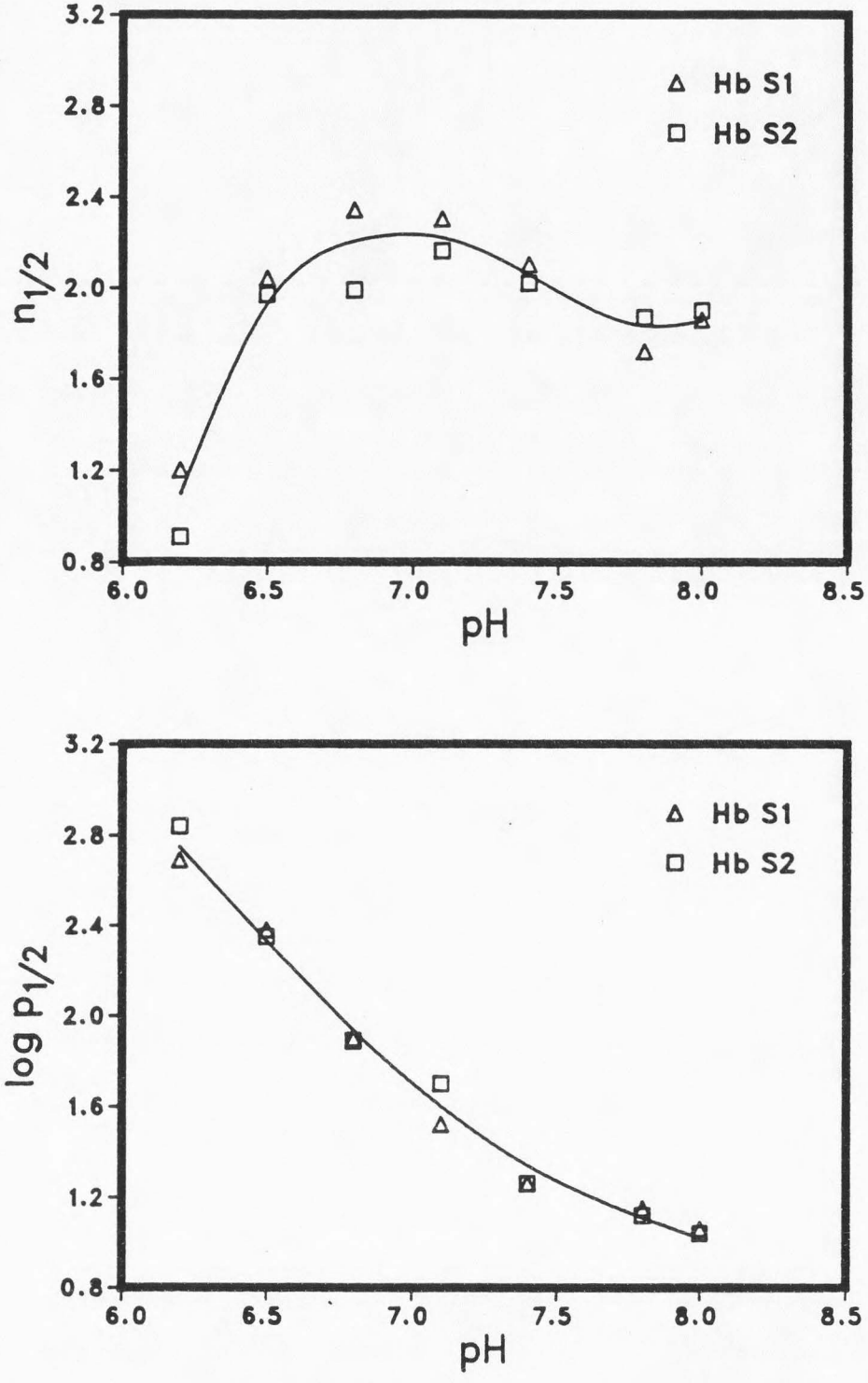


FIG. 30. **pH dependence of oxygen equilibrium parameters of Hb S1 and Hb S2.** The values of $n_{1/2}$ and log $p_{1/2}$ from the equilibrium curves for each hemoglobin as indicated. The values at pH 6.2 are estimated by extrapolation of a curve fitted by eye to the experimental data. These values, especially for $n_{1/2}$, should be regarded as approximations only.

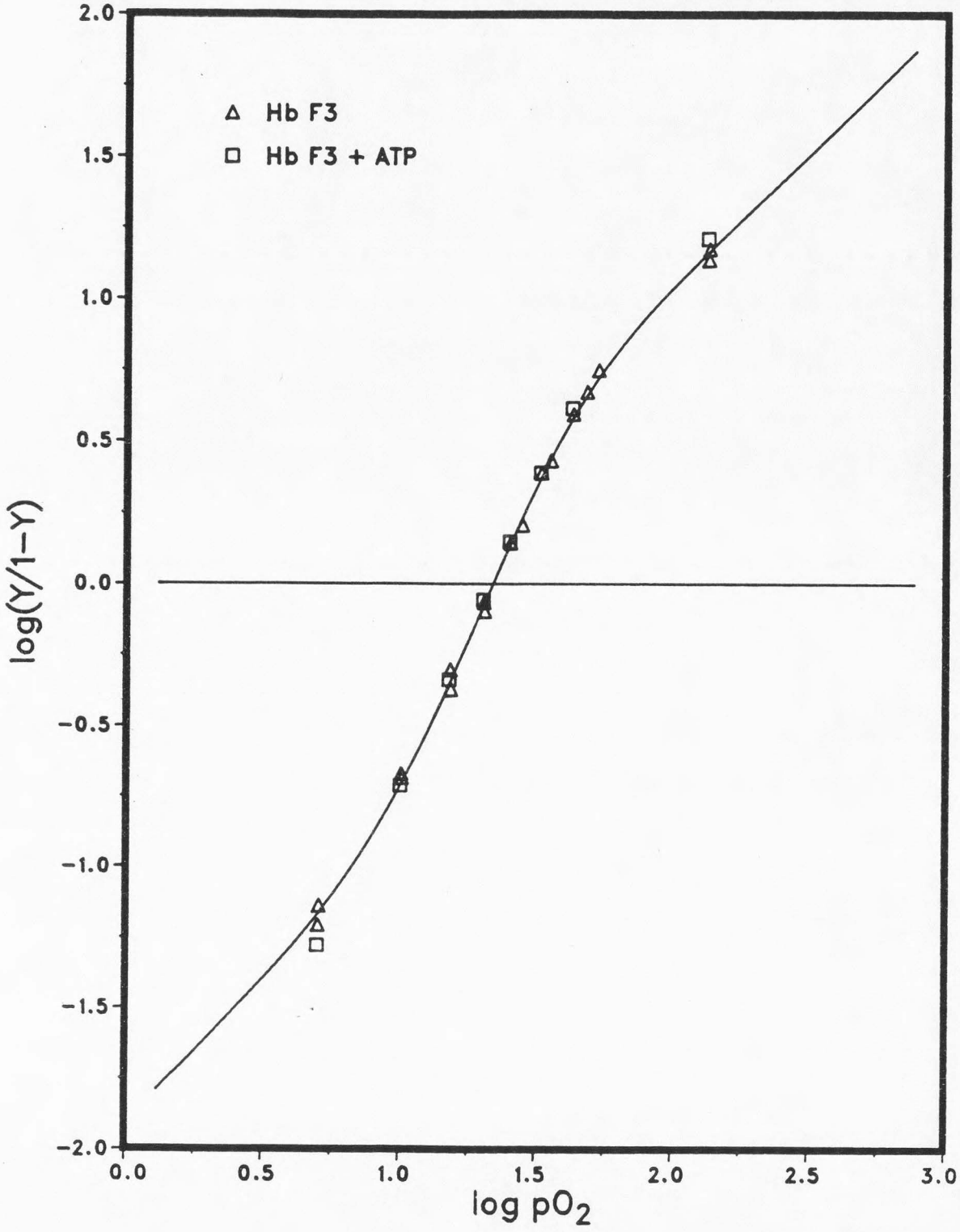


FIG. 31. **Effect of ATP on oxygen equilibrium of Hb F3.** Oxygen equilibrium curves at pH 7.1 and 20°C in the absence and presence of added ATP as indicated. Conditions as described in Materials and Methods.

ATP. A slightly larger shift is observed for both hemoglobins in the presence of GTP (Figs. 32 and 33). This small difference in the effect of ATP and GTP on these hemoglobins is also apparent from a plot of the fractional saturation of Hb S (Hb S1 + Hb S2) versus pH at constant oxygen pressure. Fig. 34 shows that the Root effect for Hb S is shifted to higher pH in the presence of these nucleotides. The hemoglobin is 50% deoxygenated at pH 6.8 in the absence of nucleotides, at pH 7.0 in the presence of ATP, and at pH 7.1 in the presence of GTP. The effects of ATP and GTP on the values of $n_{1/2}$ and log $p_{1/2}$ for each of the hemoglobins are shown in Table VII.

The temperature dependence of the oxygen equilibria of the purified hemoglobins was determined in the 10°C to 30°C range. For all of the hemoglobins except Hb S1 and Hb S2, temperature affects mainly the shape of the equilibrium curves, with only small shifts of the the curves to higher oxygen pressures as the temperature is increased. This is shown for Hb F3 in Fig. 35. In the case of Hb S1 and Hb S2, the effect of temperature on the shape of the equilibrium curve is less than for the other hemoglobins and the shift of the curve to higher oxygen pressures is more pronounced. This is shown for Hb S1 in Fig 36. The effect of temperature on $n_{1/2}$ and log $p_{1/2}$ for each of the hemoglobins is shown in Figs. 37, 38, and 39. The large value of the overall enthalpy of oxygenation (ΔH_{av}) for Hb S1 and Hb S2 as compared to the other hemoglobins is illustrated by the van't Hoff plot for Hb F3 and Hb S1 (Fig. 40). The value of ΔH_{av} obtained from the van't Hoff plot for each of the hemoglobins is given in Table VIII.

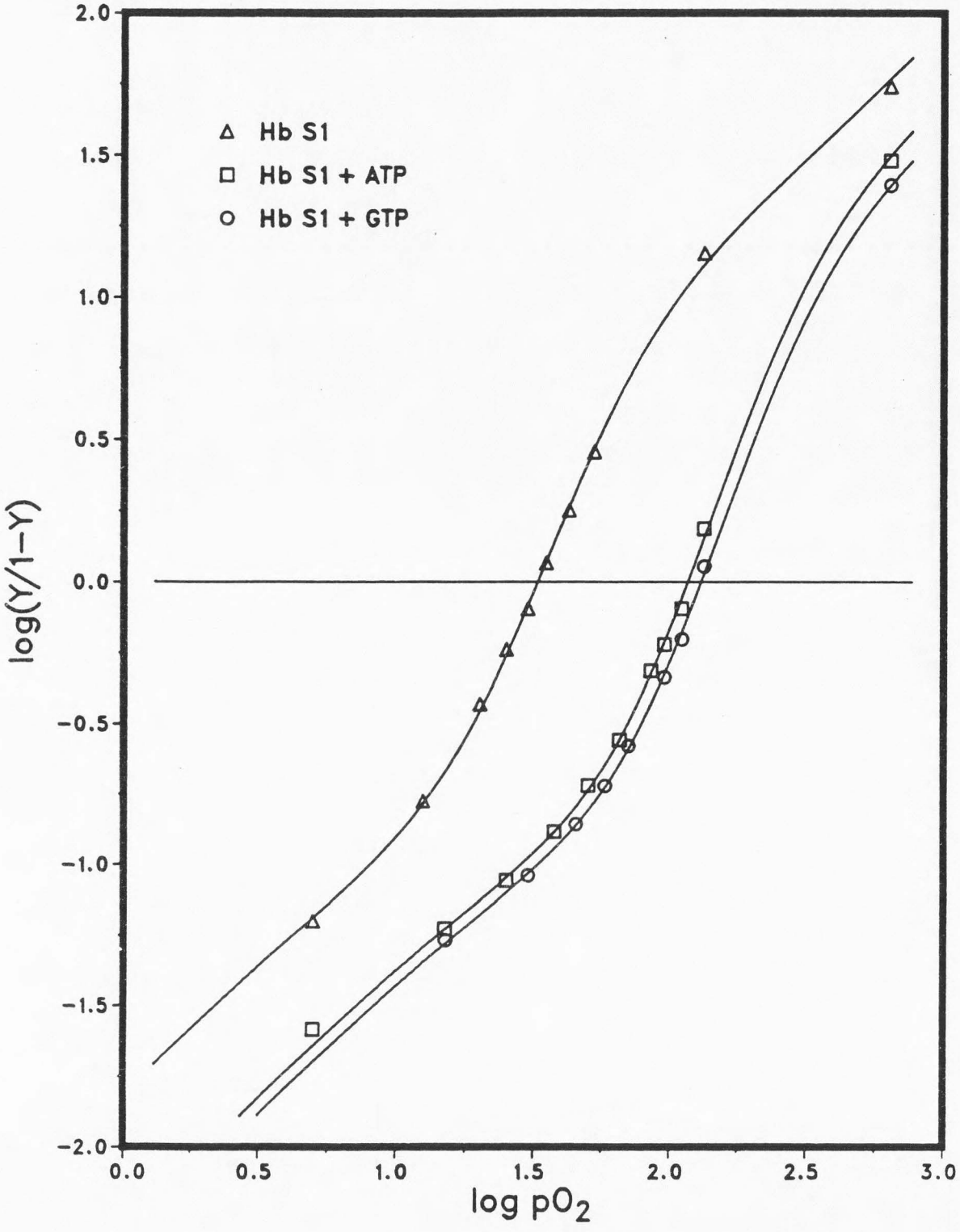


FIG. 32. **Effect of ATP and GTP on oxygen equilibrium of Hb S1.** Oxygen equilibrium curves at pH 7.1 and 20°C in the absence and presence of added ATP or GTP as indicated. Conditions as described in Materials and Methods.

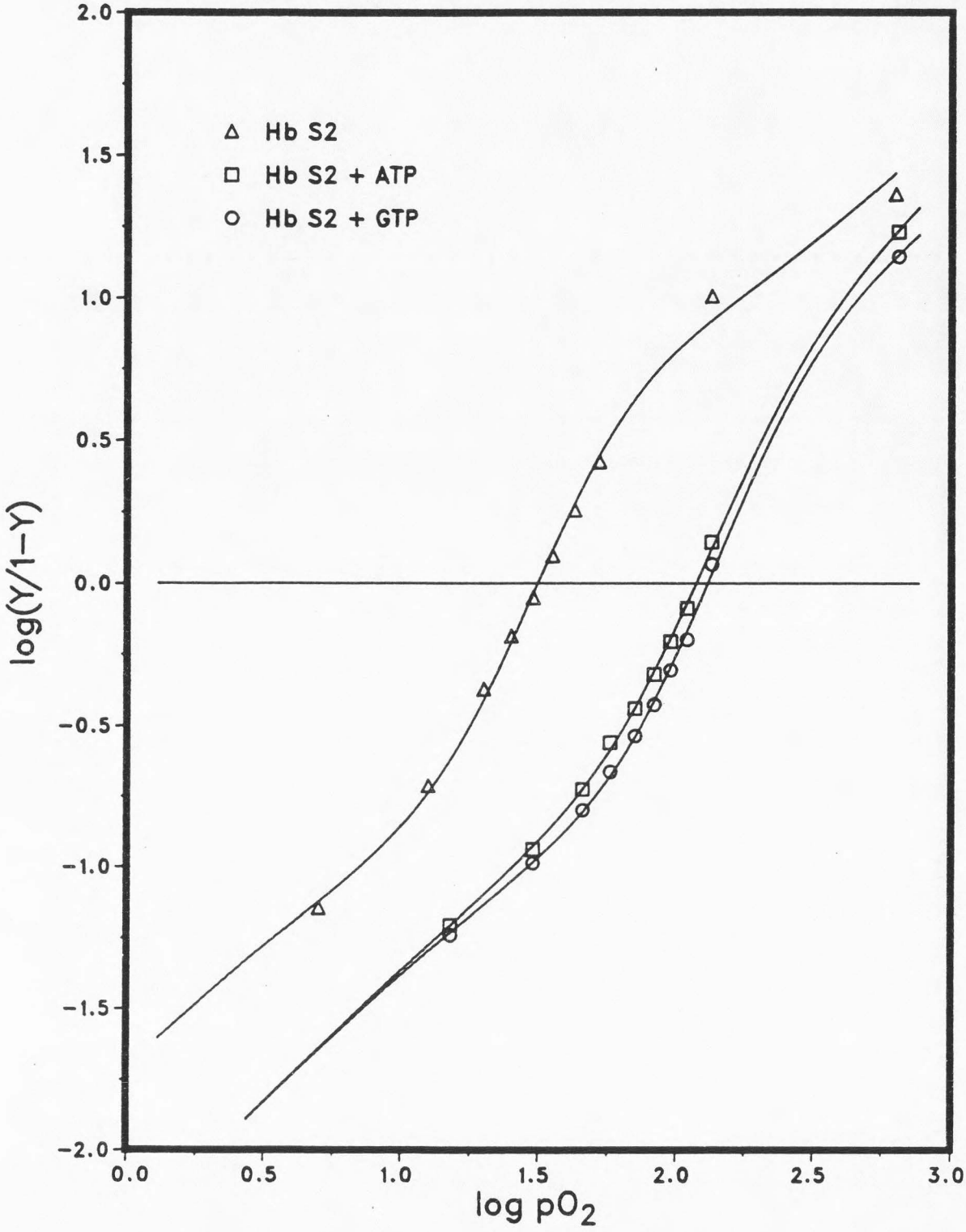


FIG. 33. **Effect of ATP and GTP on oxygen equilibrium of Hb S2.** Oxygen equilibrium curves at pH 7.1 and 20°C in the absence and presence of added ATP or GTP as indicated. Conditions as described in Materials and Methods.

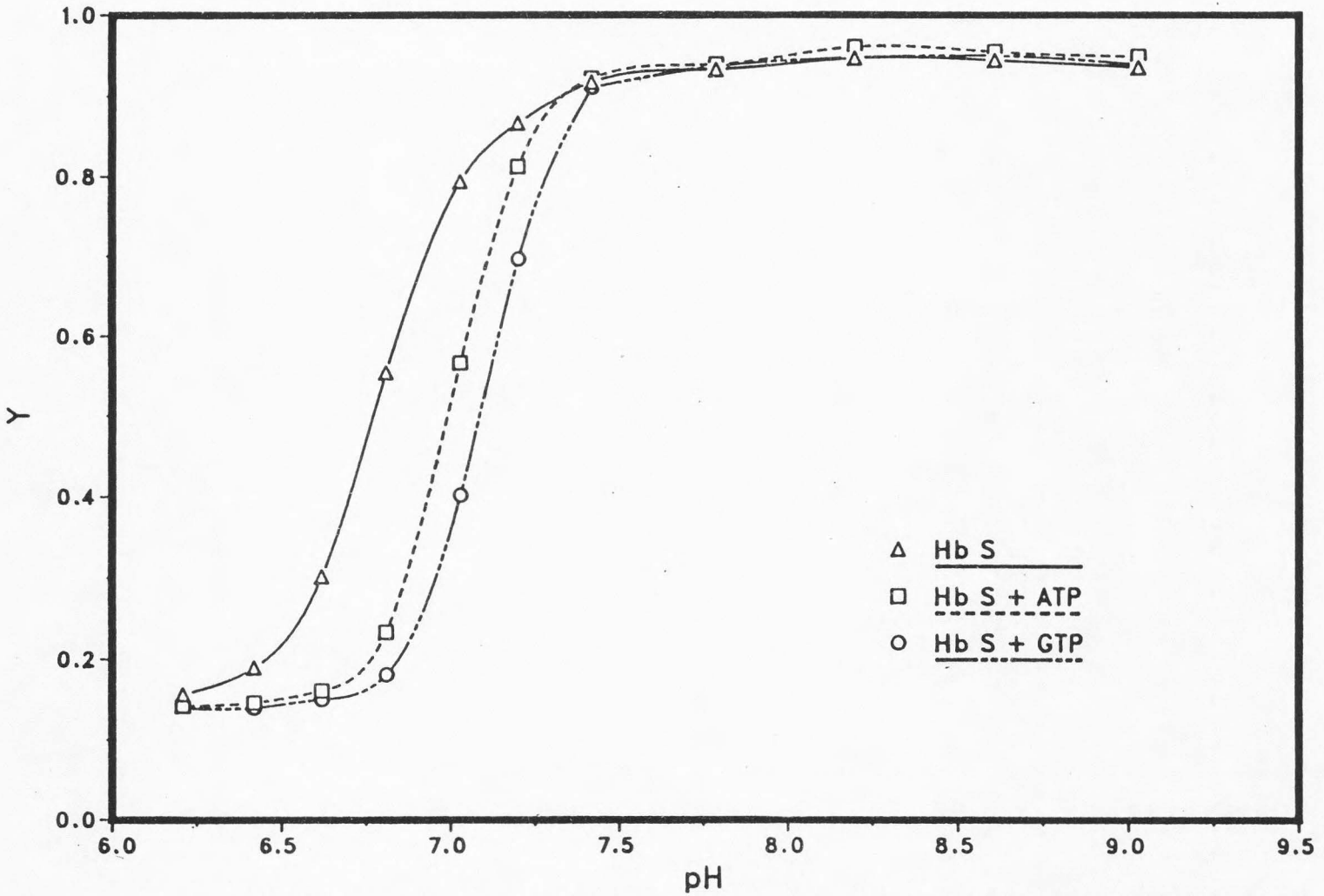


FIG. 34. **Effect of ATP and GTP on fractional saturation of Hb S as a function of pH.** Hb S (25 uM) was equilibrated at pO_2 = 134 mmHg for 5 min at 20°C without added nucleotides and with ATP or GTP (each 300 uM) as indicated. The buffers were: Taps, pH 9.0-8.2; Tes, pH 7.8-7.2; Bis-tris, pH 7.0-6.2 (all 50 mM + 0.1 M NaCl).

TABLE VII

Effect of ATP and GTP on oxygen equilibrium parameters of cutthroat trout hemoglobins

The values of $n_{1/2}$ and log $p_{1/2}$ calculated from the oxygen equilibrium curves for each hemoglobin at pH 7.1 and 20°C with the additions indicated.

	Addition:					
	None		ATP[a]		GTP[a]	
Hb	$n_{1/2}$	log $p_{1/2}$	$n_{1/2}$	log $p_{1/2}$	$n_{1/2}$	log $p_{1/2}$
F1	2.3	1.31	2.3	1.32	n d	
F2	2.3	1.33	2.4	1.33	n d	
F3	2.2	1.34	2.2	1.34	n d	
F4	2.2	1.34	2.3	1.34	n d	
M1	2.2	1.34	2.2	1.34	n d	
M2A	2.0	1.28	2.0	1.27	n d	
M2B	2.0	1.31	2.0	1.30	n d	
S1	2.3	1.52	2.5	2.07	2.5	2.12
S2	2.2	1.50	2.1	2.07	2.3	2.12

[a]ATP or GTP was added to give a nucleotide/heme ratio ~ 20.

n d, not determined.

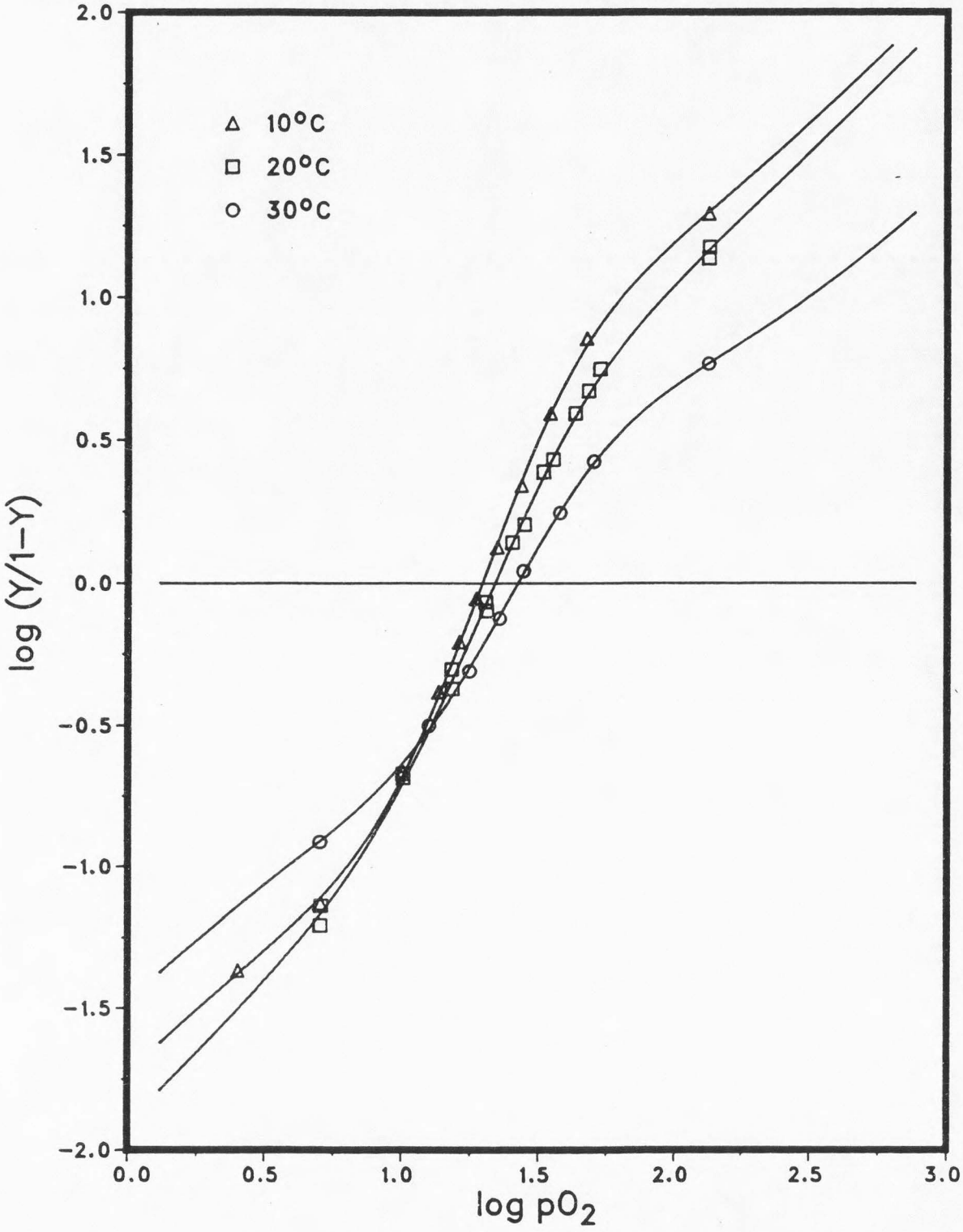


FIG. 35. **Temperature dependence of oxygen equilibrium of Hb F3.** Oxygen equilibrium curves at 10°C, 20°C, and 30°C (pH 7.1) as indicated. Conditions as described in Materials and Methods.

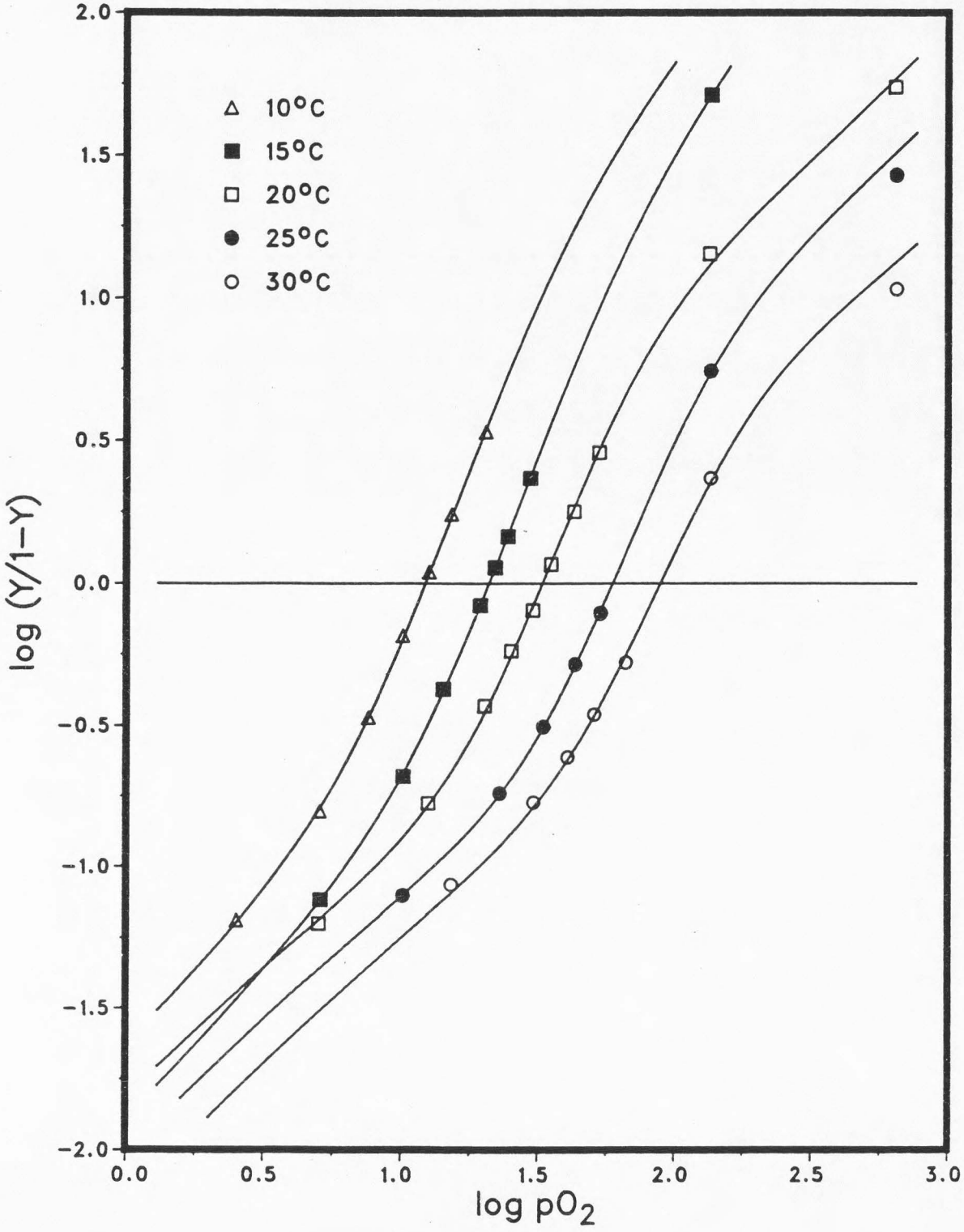


FIG. 36. **Temperature dependence of oxygen equilibrium of Hb S1.** Oxygen equilibrium curves at 10°C, 15°C, 20°C, 25°C, and 30°C (pH 7.1) as indicated. Conditions as described in Materials and Methods.

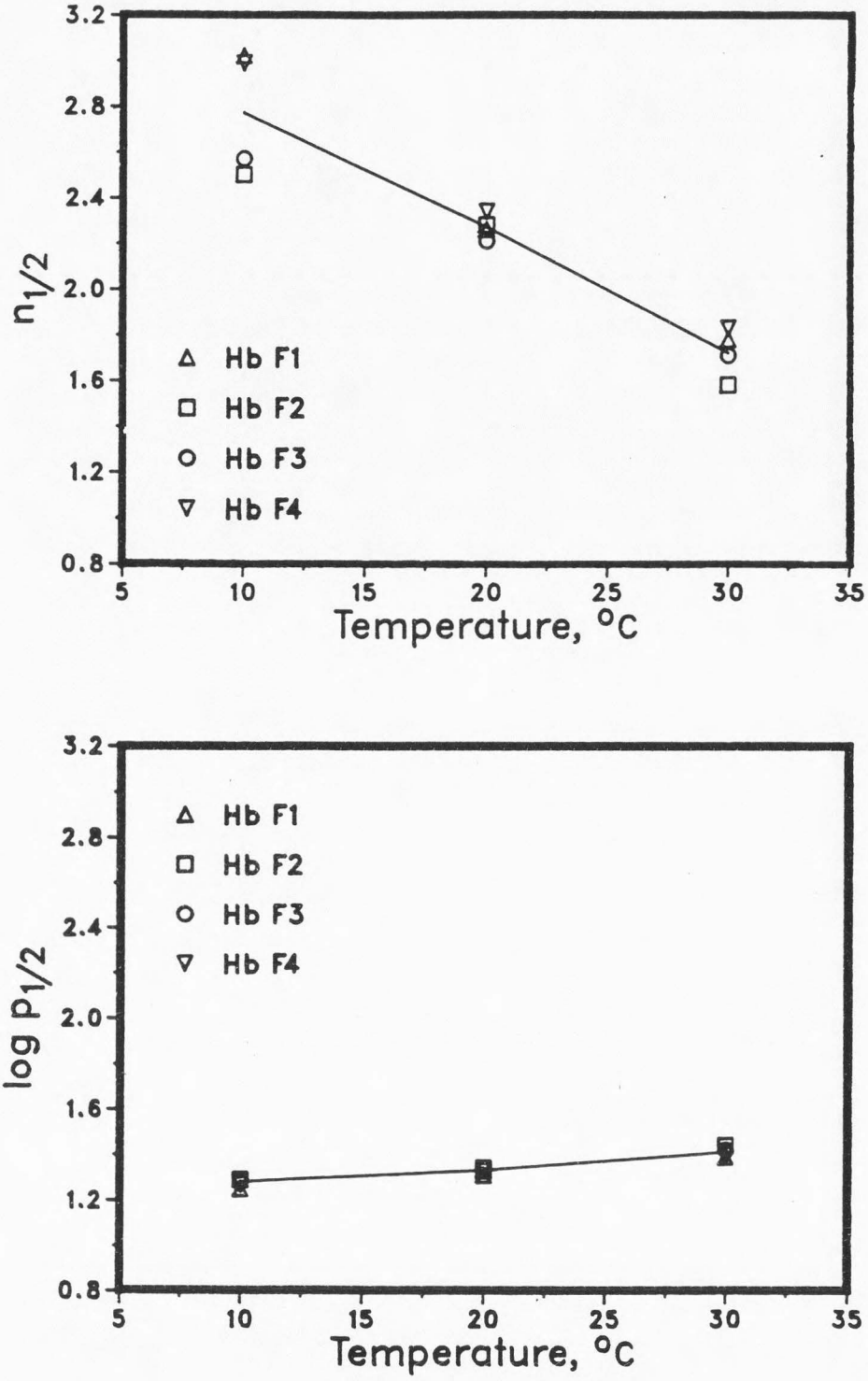


FIG. 37. **Temperature dependence of oxygen equilibrium parameters of Hb F1, Hb F2, Hb F3, and Hb F4.** The values of $n_{1/2}$ and log $p_{1/2}$ from the equilibrium curves for each hemoglobin as indicated.

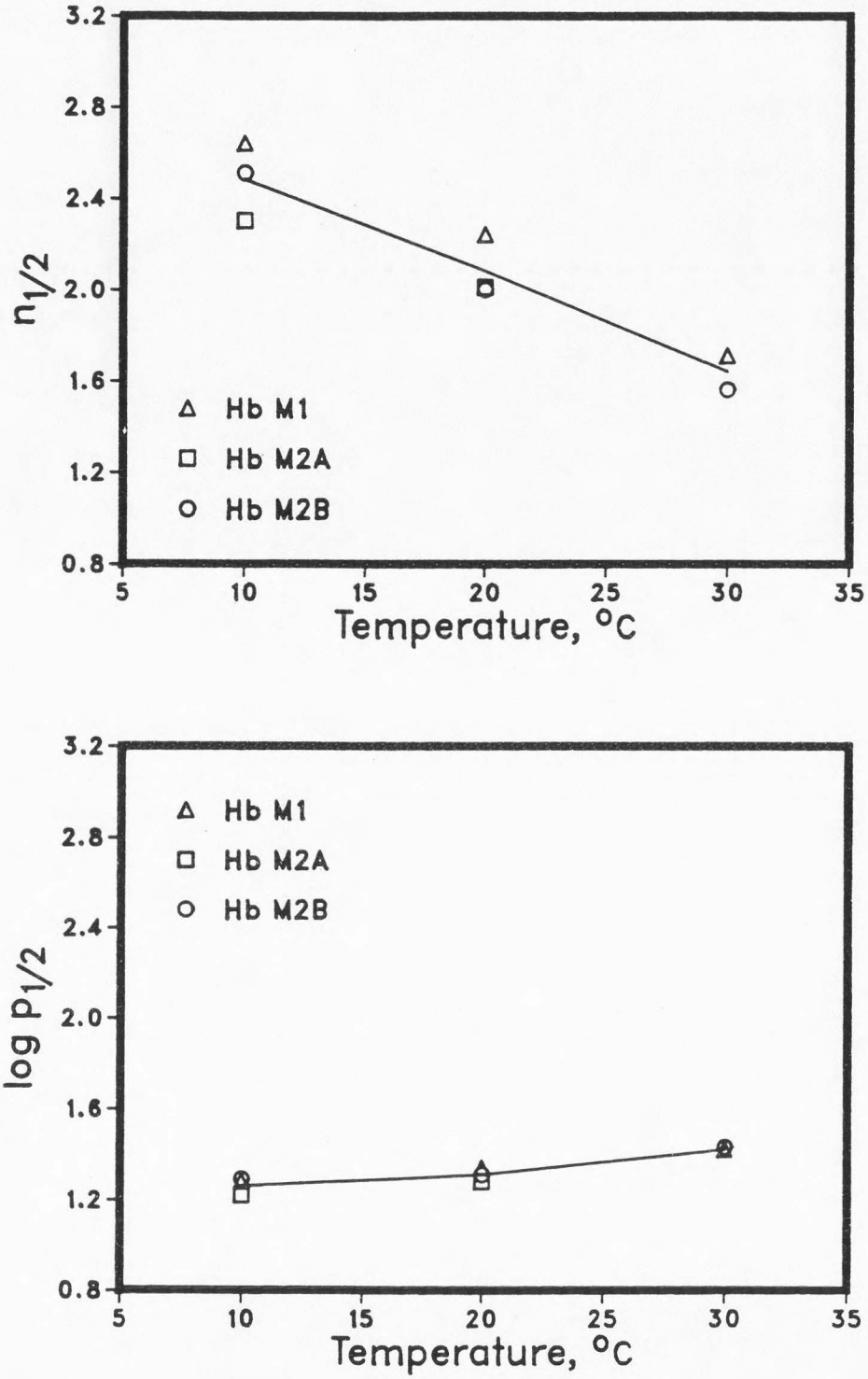


FIG. 38. **Temperature dependence of oxygen equilibrium parameters of Hb M1, Hb M2A, and Hb M2B.** The values of $n_{1/2}$ and log $p_{1/2}$ from the equilibrium curves for each hemoglobin as indicated.

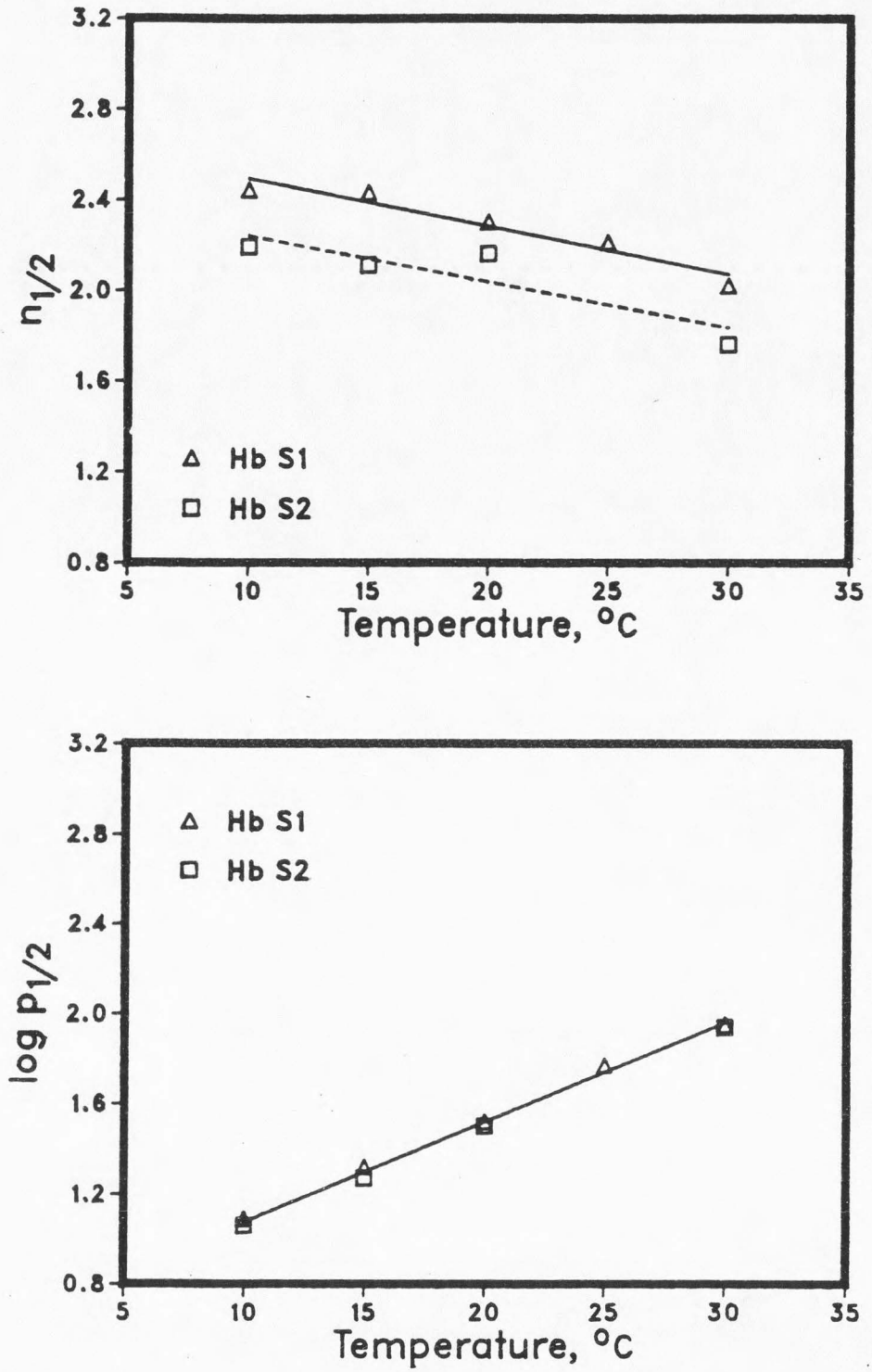


FIG. 39. **Temperature dependence of oxygen equilibrium parameters of Hb S1 and Hb S2.** The values of $n_{1/2}$ and log $p_{1/2}$ from the equilibrium curves for each hemoglobin as indicated.

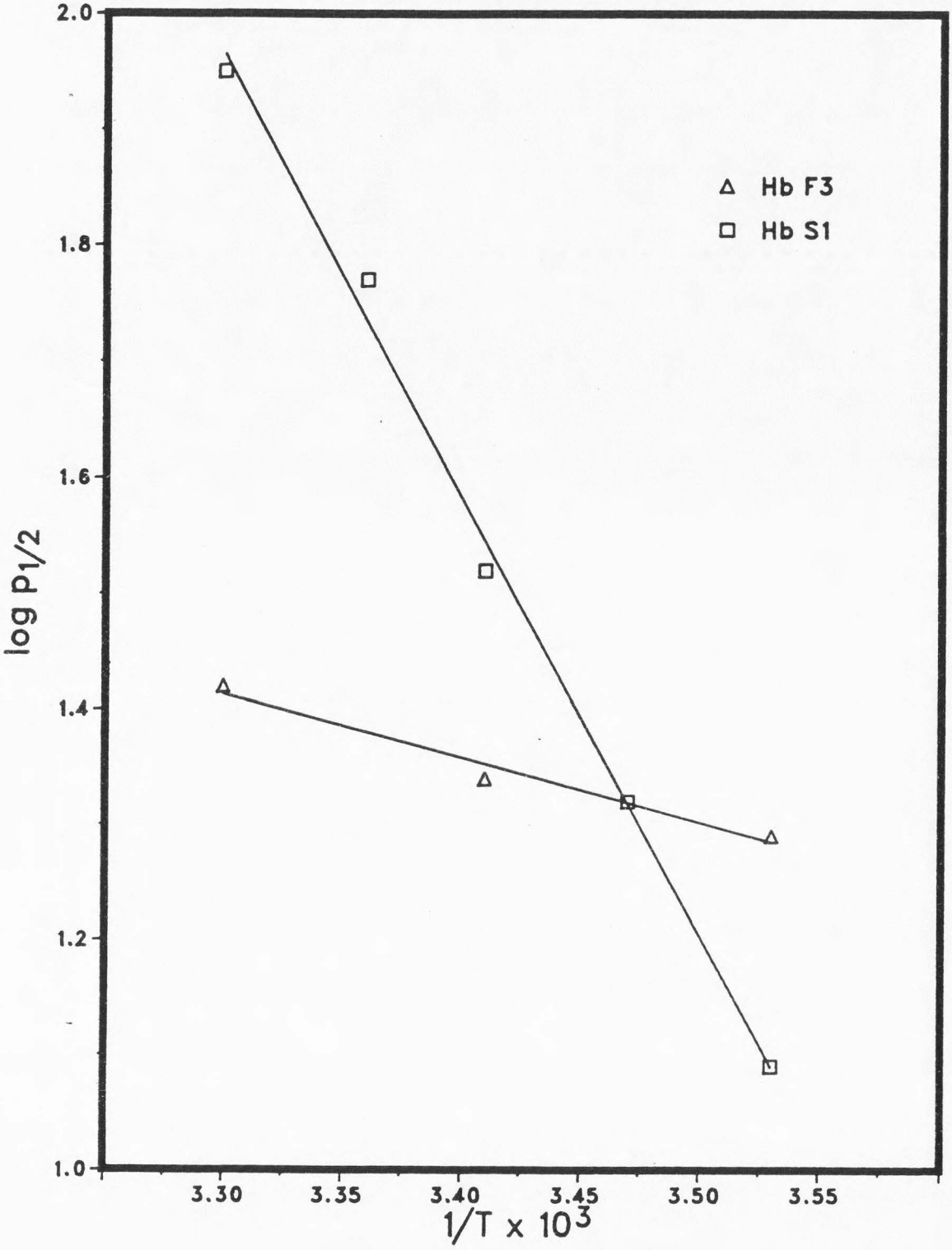


FIG. 40. **van't Hoff plot for Hb F3 and Hb S1.** The values of log $p_{1/2}$ obtained from oxygen equilibrium curves between 10°C and 30°C (pH 7.1) are plotted versus the reciprocal of the absolute temperature. ΔH_{av} is obtained from the slope of the line.

TABLE VIII

Overall enthalpy of oxygenation for cutthroat trout hemoglobins

Values of ΔH_{av} obtained from the van't Hoff plot for oxygen equilibria at pH 7.1 between 10°C and 30°C.

Hemoglobin	ΔH_{av}, kcal/mol[a]
F1	+0.4
F2	+0.2
F3	+0.6
F4	+0.6
M1	+0.4
M2A	+0.8
M2B	+0.4
S1	-14
S2	-14

[a]Corrected for the heat of solution of oxygen (-3.1 kcal/mol at 20°C).

A complete listing of the oxygen equilibrium curves determined for each hemoglobin and the values of $n_{1/2}$ and log $p_{1/2}$ obtained from these curves are given in Appendix B.

Subunit compositions of cutthroat trout hemoglobins—Initial attempts to prepare globins from the purified cutthroat trout hemoglobins by extraction of the heme in acidic acetone as described by Rossi-Fanelli et al. (134) provided poor yields (40-50%) of globins with a large portion of the acetone-precipitated protein being insoluble in distilled water. Similar results using this procedure have been reported for preparation of globins from rainbow trout hemoglobins (135). Before attempting the butanone extraction procedure suggested by these authors, several modifications of the acidic acetone procedure were investigated. It was found that mixing on a vortex mixer during the extraction as described by Chernoff and Pettit (129) rather than with a mechanical stirrer as used previously significantly improved the yields of globins. Except for Hb S1 and Hb S2, no insoluble material was visible after addition of distilled water to the acetone-precipitated protein. For Hb S1 and Hb S2, an insoluble brown residue was still observed. The yields of lyophilized globins were approximately 70% for Hb S1 and Hb S2 and 80-90% for the other hemoglobins. Hb M2A was not obtained in sufficient quantities for preparation of globin.

Initial attempts to separate the globin chains by electrophoresis gave poor results. Starch gel electrophoresis in 6 M urea with pH 8.0 barbital (129) or pH 8.1 Tris-borate-EDTA (136) buffers failed to provide any separation of the globin chains. Various

modifications of these procedures did not lead to any improvement in results.

Good separation of the globin chains was obtained by electrophoresis on polyacrylamide gels containing 6 M urea, 5% acetic acid, and 2% Triton X-100. Fig. 41a shows that all the hemoglobins applied contain two types of globin chains with very different mobilities. These two types of chains are designated α and β by comparison to the mobilities of human globin chains in the same system (130). Electrophoresis under the same conditions, but with Triton X-100 omitted from the gel (Fig. 41b) shows that the presence of a non-ionic detergent greatly enhances the separation of the globin chains.

In the gels shown in Fig. 41, cysteamine was used as the reducing agent for pre-electrophoresis. The light bands visible near the β chains for each globin (except M1) are more apparent when 2-mercaptoethanol is used as the reducing agent (data not shown). Alter and Campbell (137) have reported this same phenomenon for electrophoresis of mouse globins in this system. These bands are not visible in freshly prepared mouse globins when either reducing agent is used and therefore are probably artifacts. Based on these findings we conclude that, except for Hb M1, all the cutthroat trout hemoglobins contain a single type of β chain. Therefore, except for Hb F4 and Hb M1, all the hemoglobins appear to be composed of a single chain of each type and thus have the subunit composition $\alpha_2\beta_2$. Hb F4 and Hb M1 appear to be $\alpha\alpha'\beta_2$ and $\alpha\alpha'\beta\beta'$ tetramers, respectively. The differences in mobilities of the α and β chains from the purified hemoglobins are not sufficient to determine which chains

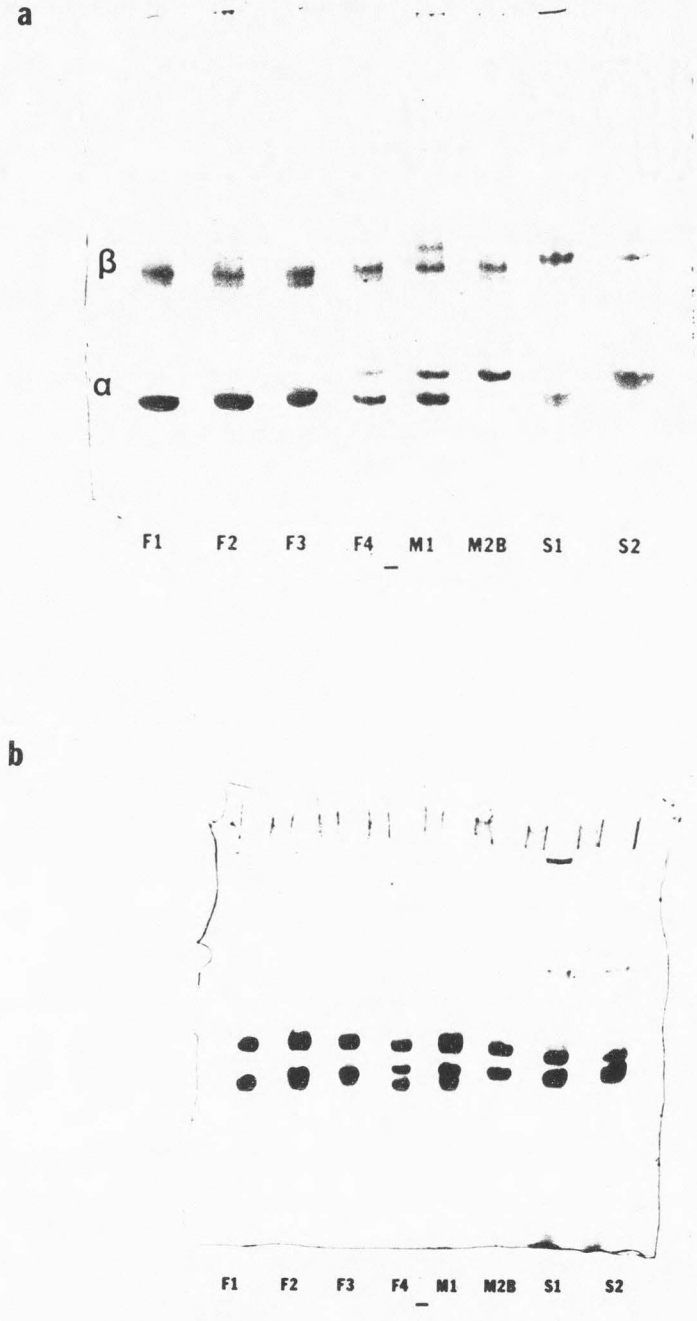


FIG. 41. **Polyacrylamide gel electrophoresis of cutthroat trout globins.** Electrophoresis of globins from the purified hemoglobins as indicated on 12% polyacrlyamide gels containing 6 M urea, 5% acetic acid, pH 2.4. (a) Gel containing 2% Triton X-100; (b) Triton X-100 omitted from the gel. The cathode is at the top of the gel.

are unique to each hemoglobin and which may be common to two or more of the hemoglobins.

The results of isoelectric focusing of cutthroat trout globins (Fig. 42) are not easily interpretable in terms of the hemoglobin subunit compositions. However, a large difference is apparent in the behavior of Hb S1 and Hb S2 as compared to the other hemoglobins in this system. The globins of Hb S1 and Hb S2 appear to have much lower isoelectric points than the other hemoglobins, a difference which is not apparent in electrophoresis of the globin chains. In both systems, the globins were applied to the gel in the same sample buffer (6 M urea, 8% acetic acid, 8% 2-mercaptoethanol), which would be expected to denature the globins and disrupt the non-covalent bonds between the subunits. The conditions to which the globins are exposed in the electrophoresis and IEF gels are similar except that in electrophoresis the pH is constant at pH 2.4, while during IEF the globins focus at their isoelectric points, presumably at pH values greater than ~ 5. Electrophoresis shows that the globins are fully dissociated in 6 M urea and 2% Triton X-100 at pH 2.4. One possible interpretation of the pattern obtained for isoelectric focusing of the cutthroat trout globins is that the dissociated globins reassociate to form tetramers or dimers as they focus at their isoelectric points. If this is true then the most prominent band for each hemoglobin in Fig. 42 may represent the tetrameric (or dimeric) globins, while the less prominent bands represent isolated subunits resulting from a small degree of dissociation.

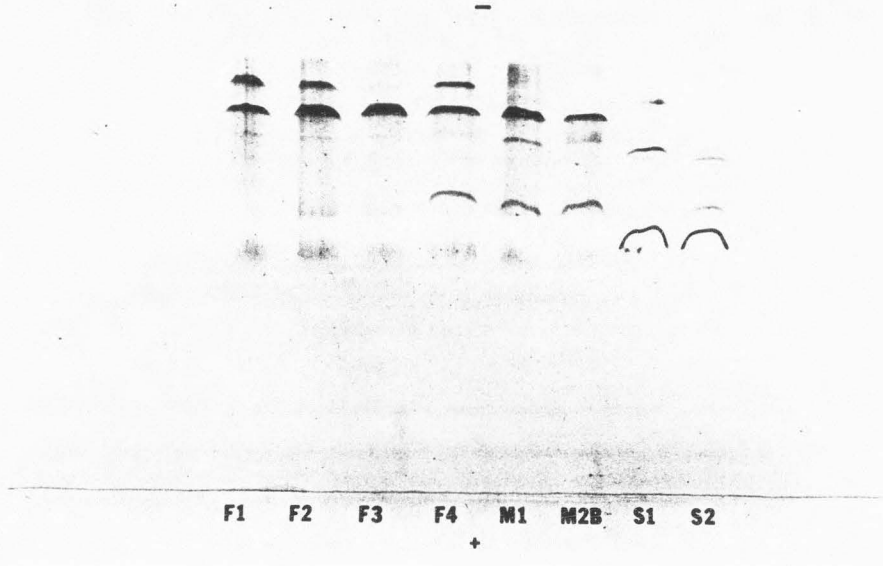


FIG. 42. **Agarose isoelectric focusing of cutthroat trout globins.** Isoelectric focusing of globins from the purified hemoglobins as indicated on 2% agarose gel containing 8 M urea, 2% Triton X-100, and 1% 2-mercaptoethanol. The cathode is at the top of the gel.

DISCUSSION

The analysis of nucleotides in the red blood cells of cutthroat trout shows that both ATP and GTP are present in these cells. Although we did not attempt to quantitate the nucleotide concentrations in relationship to the concentration of hemoglobin, ATP and GTP are probably present at relatively high concentrations. If ATP and GTP were present only in trace amounts, then we would expect to see other nucleotides (e.g. NADH) on the chromatograms, however only adenosine and guanosine nucleotides were detected. The relatively high proportion of GTP found in cutthroat trout red blood cells (ATP/GTP = 3) suggests that, in contrast to rainbow trout where GTP levels are probably too low to have much effect on hemoglobin, GTP as well as ATP may be physiologically important in the regulation of hemoglobin oxygen affinity in cutthroat trout.

No GTP could be detected in the red blood cells of rainbow trout, although ATP was present at approximately the same concentration as in red blood cells of cutthroat trout. Using essentially the same method for analysis, Weber et al. (74) found both ATP and GTP in rainbow trout red blood cells, with an ATP/GTP ratio of 9. Tetens and Lykkeboe (105) also found low levels of GTP (ATP/GTP = 7.5) in rainbow trout red blood cells by enzymatic assay. Our failure to detect any GTP in these cells may reflect differences in experimental techniques or differences in red blood cell nucleotide concentrations, either due to inherent differences in rainbow trout populations or related to the conditions under which fish were

maintained and blood was sampled.

Nine hemoglobins were isolated from blood of cutthroat trout by the purification procedure reported here. This number is intermediate between the 7 (41) and 12 (42) hemoglobins reported in previous electrophoretic studies. In this study, the system used for starch gel electrophoresis was essentially the same as that used by Braman et al. (42). They identified 6 closely spaced bands at the anodal end of their gels. Although 6 distinct bands could be distinguished several times during the course of this study, this pattern could not be consistently reproduced. The apparent complexity in this region of starch gels was comfirmed by the failure to separate these hemoglobins on preparative isoelectric focusing gels and the multiplicity of bands seen in analytical isoelectric focusing of the fractions from the broad band of hemoglobin obtained (Fig. 13a). The observation that conversion of these fractions to methemoglobin or cyanomethemoglobin prior to analytical IEF does not decrease the number of bands visible (Fig. 13b) shows that the complex pattern is not due solely to formation of deoxyhemoglobin as the hemoglobin focuses at its isoelectric point (which is low enough for deoxygenation to occur due to the Root effect). Likewise, the multiple bands are not due to oxidation intermediates, since spectra of the methemoglobin samples applied show them to be 100% oxidized.

The origin of the complexity in this region of electrophoresis and isoelectric focusing gels is an interesting question. A similar situation has been noted, but not investigated, for the anodal hemoglobins of rainbow trout (87, 93). However, since the primary

concern of this study was the cathodal hemoglobins, this phenomenon was not further investigated and this region was operationally considered to be composed of two hemoglobins, Hb S1 and Hb S2.

All the hemoglobins isolated are > 90% oxyhemoglobin and are essentially pure (with the qualification given above) as judged by analytical isoelectric focusing (Fig. 10) and starch gel electrophoresis (Figs. 11 and 14). Chromatography of the whole hemoglobin on Sephadex G-100 (Fig. 22) indicates that all the hemoglobins are tetramers with molecular weights of approximately 64,000. The cooperative oxygen binding observed for each of the isolated hemoglobins confirms this finding, since the tetramer is required for cooperativity (1, 2).

The method described here for ion-exchange chromatography is superior to the methods described previously for separation of hemoglobins of rainbow trout (89, 90, 111) in two respects. With the NaCl step elution, all the fractions, except fraction F, are effectively concentrated by this procedure. This can be seen qualititively by comparing the volume required to elute each fraction in Fig. 9 to the volume of fraction F, which is essentially equal to the volume of the sample applied. In contrast, the linear NaCl and pH gradient elutions tested and those reported previously lead to dilution of these fractions. As discussed by Schroeder and Huisman (138) for human hemoglobin, substitution of DEAE-cellulose and high molarity (0.1 M) glycine buffers for the commonly used DEAE-Sephadex gels and dilute (5-50 mM) Tris buffers also contributes to the improved separation reported here. The step elution method also results in a considerable decrease in the time required to run the column. By

beginning each step of the elution when the previous fraction is well separated from the hemoglobin bound at the top of the column (but not yet eluted from the column), the separation could be completed in 4-5 hours.

The ability to concentrate the hemoglobins during chromatography is an advantage since concentration by ultrafiltration causes an increase in the proportion of methemoglobin in the samples. In an attempt to concentrate fraction F during ion-exchange chromatography, chromatography on the cation exchange resins CM-Sepharose CL-6B and CM-cellulose was investigated. No binding of cutthroat trout hemoglobins to these resins was observed in the pH 7.0-8.2 range (phosphate and glycine buffers). The concentration effect also makes it possible to resolve hemoglobins such as Hb M2A and Hb M2B which are present in the hemolyzate at low concentrations. These hemoglobins are not apparent in elution profiles when continuous gradient elution is used. The saving in time is important as it was found that even when hemoglobin solutions were kept at $4^{o}C$ and at pH 7.7-8.2, increasing oxidition to methemoglobin became apparent within 8-10 days after blood was obtained. With the purification procedure described the purified hemoglobins could be obtained within 3 days of taking blood.

A comparison of the hemoglobins of cutthroat and rainbow trout by starch gel electrophoresis (Fig. 43) shows that the patterns are identical except for the presence of 3 additional bands in the cutthroat trout hemolyzate, as described previously (42). These 3 bands correspond to Hb F1, Hb F2, and Hb F4 (refer to Figs. 11 and

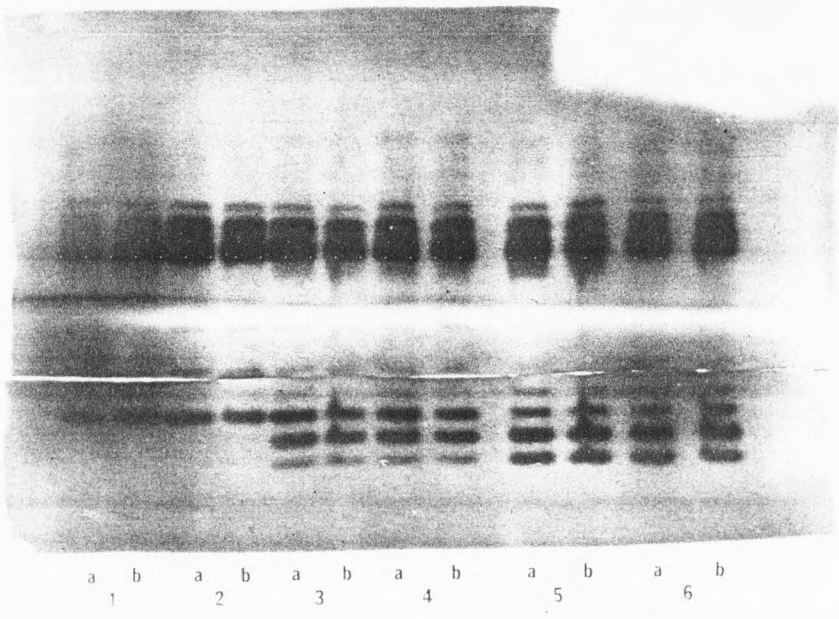


FIG. 43. **Starch gel electrophoresis of hemoglobins from rainbow and cutthroat trout.** Crude hemolyzates from two rainbow trout (lanes 1 and 2) and four cutthroat trout (lanes 3-6). Each sample was applied twice (a and b). Conditions as described in Materials and Methods.

14 for starch gel electrophoresis of the purified hemoglobins). Hemoglobins with mobilities identical to Hb F3, Hb M1, Hb M2B, Hb S1, and Hb S2 are present in the rainbow trout hemolyzate. Although it is tempting to suggest that the most anodal band (just above the two prominent bands which represent Hb S1 and Hb S2) is Hb M2A, there is no evidence for this assignment.

Braman (111) compared the electrophoretic patterns of rainbow trout hemoglobins obtained by the electrophoretic system described here and by the system used by Giovenco et al. (87) who have studied these hemoglobins extensively. His results show that the hemoglobins isolated by this group from rainbow trout, Hb I, Hb II, Hb III, and Hb IV, correspond to Hb F3, Hb M1, Hb S1, and Hb S2, respectively, from cutthroat trout. This conclusion is supported by a comparison of the known properties of the corresponding hemoglobins, Hb I and Hb F3; and Hb IV and Hb S2 (Table IX). The large difference in the overall enthalpy of oxygenation (ΔH_{av}) for Hb IV and Hb S2 may be due to the fact that the oxygen equilibrium curves of Hb IV were measured in 0.2 M phosphate buffer. At this high concentration, phosphate is a fairly strong heterotropic effector of Hb IV. At 20°C, the value of log $p_{1/2}$ is 1.60 in 50 mM Bis-tris, pH 7.1 (98) and 2.30 in 0.2 M phosphate, pH 7.1 (89). The positive enthalpy associated with the release of bound phosphate upon oxygen binding will result in a lower apparent value for the enthalpy of oxygenation. Thus the hemoglobins from rainbow and cutthroat trout with corresponding electrophoretic mobilities have similar oxygen binding properties. These findings suggest that these hemoglobins probably have very similar structures in these two species of trout.

TABLE IX

Comparison of some properties of hemoglobins from rainbow and cutthroat trout

Data for rainbow trout hemoglobins (Hb I and Hb IV) are from references 89 and 93. Data for cutthroat trout hemoglobins (Hb F3 and Hb S2) are from this study.

	Hb I	Hb F3	Hb IV	Hb S2
Isoelectric point	8.2	8.15	6.2-6.5	6.45
Subunit type	$\alpha_2\beta_2$	$\alpha_2\beta_2$	$\alpha_2\beta_2$	$\alpha_2\beta_2$
Oxygen equilibrium:				
Root effect	no	no	yes	yes
Affected by ATP	no	no	yes	yes
ΔH_{av}, kcal/mol	~0	+0.6	-7[a]	-14[b]

[a] In 0.2 M phosphate, pH 7.1.

[b] In 50 mM Tes, 0.1 M NaCl, pH 7.1.

As described above, starch gel electrophoresis shows the presence of a hemoglobin in rainbow trout hemolyzates with a mobility identical to that of Hb M2B from cutthroat trout. Analytical isoelectric focusing of rainbow trout whole hemoglobin under the same conditions as used for cutthroat trout hemoglobins shows the presence of two minor bands which focus in positions similar to Hb M2A and Hb M2B from cutthroat trout (Fig. 44). Thus, by the methods used here, we detect a total of 6 hemoglobins in rainbow trout, corresponding to Hb F3, Hb M1, Hb M2A, Hb M2B, Hb S1, and Hb S2 from cutthroat trout. The failure of Binotti et al. (89) and Lau et al. (90) to detect the two minor components may be ascribed to the lower resolving power of the linear gradient elutions used for ion-exchange chromatography and the higher detection limits of starch gel electrophoresis as compared to the techniques used in this study (step gradient elution and analytical isoelectric focusing).

The tonometric method used here to determine oxygen equilibrium curves differs from the methods most commonly used in one important aspect. In the standard procedure (124, 125), the value of Y at each step in the oxygenation is calculated from absorbance measurements at a single wavelength. This calculation assumes that the fully oxygenated sample represents 100% oxyhemoglobin, that the deoxygenated sample represents 100% deoxyhemoglobin, and that the changes in absorbance in going from one state to another are linearly related to the degree of oxygenation. Although this last assumption has been shown to be valid (2, 125), the validity of the first two will depend on the particular technique used for oxygenation and

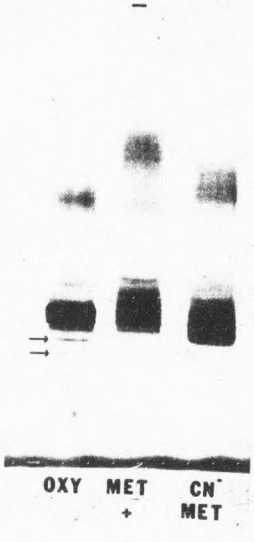


FIG. 44. **Agarose isoelectric focusing of rainbow trout hemoglobins.** Whole hemoglobin (after G-25 chromatography) was applied as oxyHb (OXY), as metHb (MET), and as cyanometHb (CN^- MET). Minor components corresponding to Hb M2A and Hb M2B from cutthroat trout are indicated.

deoxygenation and on the oxygen affinity of the specific hemoglobin solution being used. Because the hemoglobins of active fishes such as trout generally have low oxygen affinities (59), they may not be fully saturated when equilibrated with air, or under some conditions, even with oxygen at atmospheric pressures. This will clearly be the case for Root effect hemoglobins at low pH. The net result of assuming 100% saturation when the hemoglobin is not fully saturated with oxygen is that the oxygen equilibrium curve, especially the upper portion, is shifted to the left. If the difference between the actual and assumed saturation is large enough, errors will be introduced in the parameters derived from the Hill plot. The apparent values of $n_{1/2}$ and log $p_{1/2}$ will be higher and lower, respectively, than the actual values.

In the method used here, the percent saturation with oxygen at each point in the oxygen equilibrium curve was determined directly from the concentrations of the three hemoglobin species (oxyHb, deoxyHb, and metHb) calculated from absorbance measurements at three wavelengths. Therefore, the need to assign absorbance measurements representing 100% deoxyHb and 100% oxyHb are eliminated. These calculations show that in most of the equilibrium measurements reported here, the hemoglobins are only 92-97% saturated with oxygen when equilibrated with air (pO_2 ~ 135 mmHg). These values could reflect the low affinity of these hemoglobins under the conditions tested, or may be due to the use of inappropriate extinction coefficients which result in an underestimation of the oxyhemoglobin concentration. If the latter is true, then we would expect to see a difference in the oxygen equilibrium curve determined by the two

methods when the absorbances of solutions approximating 100% oxyHb and 100% deoxyHb are known. In the determination shown in Fig. 45 (Hb S1 at pH 8.0 and 20°C), the final equilibration was with oxygen at 640 mmHg. The calculated hemoglobin concentrations indicate that at this point the sample was >99% oxyHb while after deoxygenation the sample was >98% deoxyHb. For analysis of the equilibrium data by the single wavelength method, the absorbance at 576 nm of these samples was taken to represent 100% oxyHb and 100% deoxyHb, respectively, and the %oxyHb at each intermediate step calculated from the A_{576} at each step by difference. The excellent agreement between the points on the equilibrium curve calculated by this method and by the use of the extinction coefficients for human hemoglobin to determine the hemoglobin concentrations shows that the application of these extinction coefficients does not introduce any significant error into these measurements.

Based on this finding and on the observed spectral properties of cutthroat trout hemoglobins, we conclude that the use of extinction coefficients for human hemoglobin in determination of the oxygen equilibria of cutthroat trout hemoglobins is valid. The method described, utilizing absorbance measurements at three wavelengths, is superior to the single wavelength method since errors due to incomplete saturation with oxygen are eliminated. Also, oxidation of the hemoglobin can be monitored at each step and the run terminated if methemoglobin levels rise too high. As many fish hemoglobins have been reported to have lower oxygen affinities and to be more readily oxidized than human hemoglobin (59), this

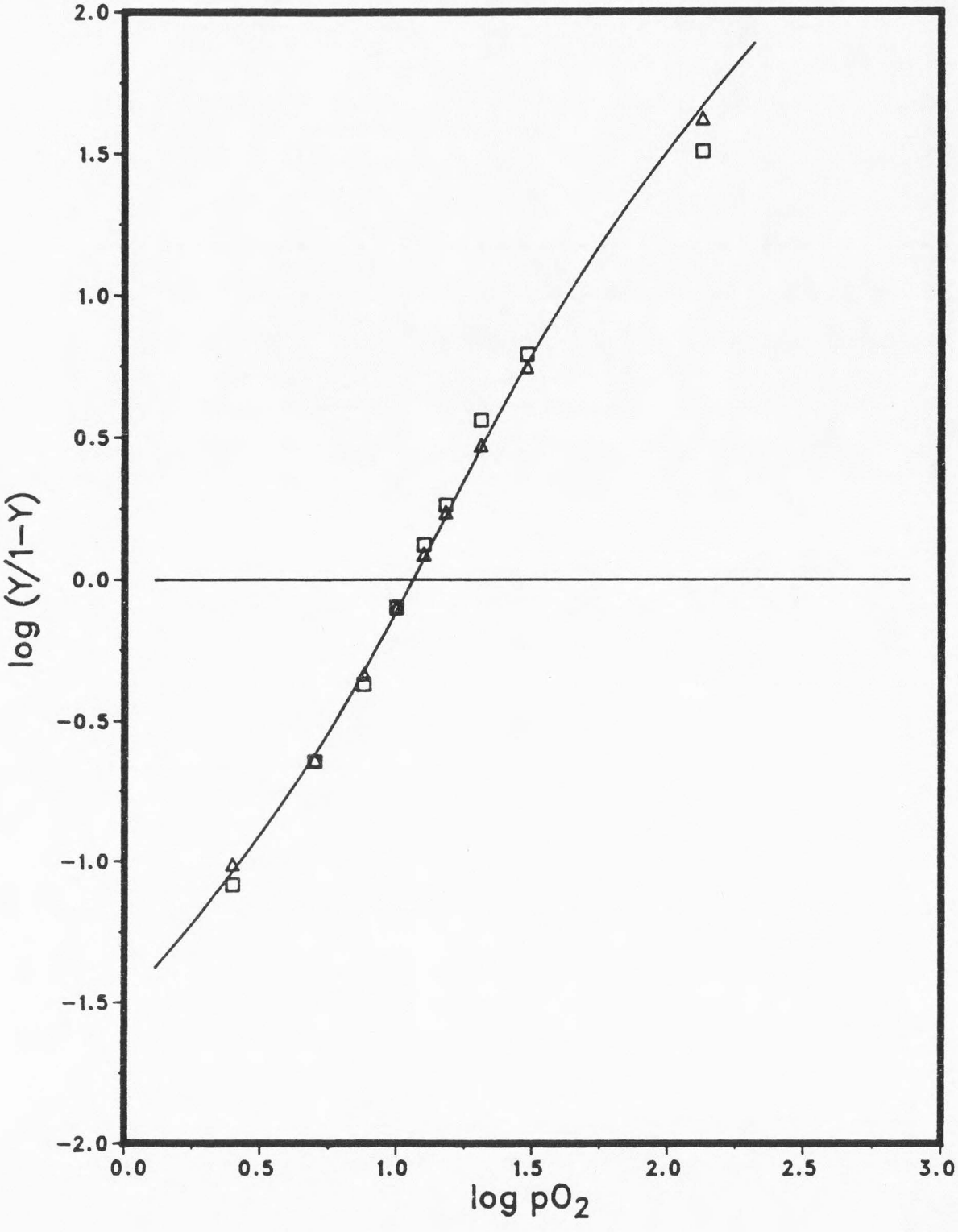


FIG. 45. **Comparison of oxygen equilibrium data analyzed by two methods.** Data for the oxygen equilibrium curve of Hb S1 at pH 8.0 and 20°C was analyzed by the single wavelength method (□) from the absorbance at 576 nm, and by the method described in Materials and Methods (Δ) based on the absorbance at 560, 576, and 630 nm.

method should be advantageous in oxygen equilibrium measurements of hemoglobins of other fishes. This method is also less sensitive to other sources of error in absorbance measurments such as concentration of the hemoglobin due to evaporation during the measurements, small changes in spectrophotometer response, or somewhat high or low absorbance readings which may occur when the bulky and heavy tonometer is slightly misaligned in the cell holder.

Of the nine hemoglobins isolated from cutthroat trout, seven (Hb F1, Hb F2, Hb F3, Hb F4, Hb M1, Hb M2A, and Hb M2B) have very similar oxygen binding properties. Hb M2A and Hb M2B are distinguished from the other five hemoglobins in this group based on the anodal mobility of Hb M2B on starch gels (the mobility of Hb M2A in this system is not known) and the low apparent isoelectric points of these proteins on isoelectric focusing gels. These two minor hemoglobins are discussed separately later. The remaining five hemoglobins are designated as cathodal hemoglobins, although Hb M1 actually migrates slightly towards the anode on starch gels. As shown above, Hb F3 and Hb M1 correspond to Hb I and Hb II of rainbow trout. Hb F1, Hb F2, and Hb F4 appear to be the only hemoglobins of cutthroat trout which do not correspond to hemoglobins found in rainbow trout.

The cathodal hemoglobins of fishes generally have oxygen equilibria which are less sensitive to heterotropic effectors than those of anodal hemoglobins. Within this context however, cathodal hemoglobins from different fishes display a variety of oxygen binding properties. For example, pH dependences ranging from normal Bohr effects to reverse Bohr effects have been reported. Some cathodal

hemoglobins are affected by organic phosphates to various extents, while others appear to be completely independent of these effectors.

Given this variety, we expected to observe some differences in the oxygen binding properties of the five cathodal hemoglobins of cutthroat trout due to interactions with heterotropic effectors. It appears however, that these hemoglobins all have essentially identical oxygen binding properties, which in each case appear to be independent of the two heterotropic effectors tested, protons and ATP. At pH 7.1 and 20°C, Hb F1, Hb F2, Hb F3, Hb F4, and Hb M1 have similar oxygen affinities (log $p_{1/2}$ = 1.31-1.34) and cooperativities ($n_{1/2}$ = 2.2-2.3), and these parameters are independent of pH in the pH 6.2-8.0 range. Saturating levels of ATP at pH 7.1 have no effect on either the affinity or the cooperativity of any of the cathodal hemoglobins. Thus, these five hemoglobins resemble Hb I and Hb II of rainbow trout in the total absence of heterotropic effects due to protons and ATP. Due to the absence of any effect of ATP, other organic phosphates were not tested.

The oxygen equilibria of the five cathodal hemoglobins also have similar temperature dependences, with the oxygen affinity showing a small decrease with increasing temperature. All appear to be similar to Hb I of rainbow trout (99), having enthalpies of oxygenation which change with the degree of saturation and overall enthalpies of oxygenation ~ 0. The value of log $p_{1/2}$ for these hemoglobins is only slightly increased as temperature increases from 10°C to 30°C. The values for the overall enthalpy of oxygenation obtained from the van`t Hoff plot range from + 0.2 to + 0.6

kcal/mol. Given the estimated error in these measurements (approximately +/- 1 kcal/mol), the overall enthaply of oxygenation for these hemoglobins are essentially equivalent and ~ 0. In contrast to the small temperature dependence of the oxygen affinity of the cathodal hemoglobins, the shape of the oxygen equilibrium curve for these hemoglobins is fairly strongly affected by temperature. This is reflected in the decrease in $n_{1/2}$ as temperature increases. For Hb F3, $n_{1/2}$ decreases from 2.6 at 10°C to 1.7 at 30°C. The dependence of the shape of the oxygen equilibrium curve indicates that the enthalpy of oxygenation is dependent on the degree of saturation with oxygen, with $\Delta H \sim 0$ at $Y = 0.5$ and becoming more negative as Y increases. This same behavior has been demonstrated for Hb I from rainbow trout (100).

Based on these results it appears that none of the five cathodal hemoglobins of cutthroat trout have any significantly different oxygen binding properties which would confer a physiological advantage to the fish. It is possible that these hemoglobins may be differently affected by two of the known physiological effectors which we have not studied, Cl^- and CO_2. However, given the degree of functional similarity observed and the fact that Cl^- and CO_2 do not appear to be strong heterotropic effectors of fish hemoglobins (24, 46, 98), we would expect any differences to be small. Therefore, if these multiple cathodal hemoglobins are physiologically important, it is probably not as a result of differences in their oxygen transport properties.

As expected, the two anodal hemoglobins, Hb S1 and Hb S2 display oxygen binding properties markedly different from those of

the cathodal hemoglobins. The oxygen equilibria of both components are strongly pH dependent with the values of log $p_{1/2}$ increasing from 1.04 and 1.06 at pH 8.0 to 2.7 and 2.8 at pH 6.2 for Hb S1 and Hb S2, respectively. The cooperativity for both hemoglobins is maximal near pH 7 ($n_{1/2}$ ~ 2.3) and decreases sharply as the pH is lowered ($n_{1/2}$ ~ 1 at pH 6.2). Both ATP and GTP cause a large decrease in the oxygen affinity of these hemoglobins. The magnitude of this decrease is identical for both hemoglobins, and in both cases GTP has a slightly greater effect than ATP. The affinity of both Hb S1 and Hb S2 for oxygen is strongly temperature dependent, corresponding to an overall enthalpy of oxygenation of -14 kcal/mol for both hemoglobins at pH 7.1. The decrease in the value of $n_{1/2}$ from 10°C to 30°C for the anodal hemoglobins is approximately half as large as for Hb F3 (0.4 versus 0.9), indicating that ΔH is less dependent on the degree of saturation with oxygen for these hemoglobins than for the cathodal hemoglobins.

The observed oxygen binding properties of Hb S1 and Hb S2 of cutthroat trout are generally in good agreement with comparable measurements for Hb III and Hb IV, the corresponding hemoglobins of rainbow trout (90, 98, 102). As reported for these hemoglobins, the two anodal hemoglobins of cutthroat trout appear to have very similar or identical functional properties. One possible difference in the oxygen binding properties of these two hemoglobins is that both nucleotides cause a small increase in the cooperativity for Hb S1 which is not evident for Hb S2. Given the experimental errors in the determination of $n_{1/2}$ this difference may be more apparent than

real. Also the values of $n_{1/2}$ for Hb S1 appear in general to be slightly higher than for Hb S2.

As shown previously in rainbow trout, the cathodal and anodal hemoglobins of cutthroat trout have very different oxygen binding properties. These two types of hemoglobins presumably serve different physiological requirements. We have found that within each type, the multiple hemoglobins (five cathodal and two anodal) of cutthroat trout have very similar oxygen binding properties. The 'benefit' to the fish in synthesizing these multiple hemoglobins is not readily apparent. They may have important functional differences which have not been detected, or may possibly be beneficial for some reason unrelated to their oxygen binding properties. The environment in which the hemoglobins function in vivo is very different than the conditions under which the oxygen equilibria reported here were determined, and interactions between the hemoglobins and with other components in the red blood cell could induce functional differences not apparent in the relatively dilute solutions of the purified hemoglobins in vitro. Of course it is possible that the presence of these multiple hemoglobins constitutes neither an advantage nor a disadvantage to the fish, ie., the presence of these hemoglobins may be selectively neutral.

It has been suggested that multiple hemoglobins with similar functional properties may increase the total solubility of hemoglobin in the red blood cell and thus allow a higher potential oxygen carrying capacity per cell (25, 79). Presumably, a mixture of hemoglobins with different surface characteristics would have a lesser tendency to crystallize than a single hemoglobin at equal

concentration. According to this hypothesis the functionally identical hemoglobins should have different surface properties. Tan-Wilson et al. (91) have probed the surface properties of the hemoglobins of rainbow trout by using antibodies to the purified hemoglobins. Two methods of analysis (complement fixation and quantitative precipitation) show that the two cathodal hemoglobins (Hb I and Hb II) react identically with rabbit anti-Hb I antibodies. Similarly, the two anodal hemoglobins (Hb III and Hb IV) are indistinguishable in their reaction with rabbit anti-Hb IV antibodies. Thus it appears that Hb I and Hb II have very similar surface properties, as do Hb III and Hb IV. These findings suggest that the multiple hemoglobins of each type (cathodal and anodal) in rainbow trout are probably not important in increasing hemoglobin solubility in the red blood cell. Similar immunological methods applied to the cathodal hemoglobins of cutthroat trout might show whether this is also true for these hemoglobins.

From the positions of Hb M2A and Hb M2B in the elution profile of the DEAE-cellulose column it appears that these hemoglobins have isoelectric points intermediate between Hb M1 (pI = 7.00-7.15) and Hb S1 (pI = 6.55). Starch gel electrophoresis also indicates that the pI of Hb M2B is intermediate between those of Hb M1 and Hb S1. However, these hemoglobins consistently focused as the most anodal bands on agarose isoelectric focusing gels. By isoelectric focusing, Hb M2B has an isoelectric point of 5.9 and Hb M2A appears to have a slightly lower pI. The reasons for the observed differences in the behavior of these two hemoglobins in these three separaration

methods is not known. Apparently, specific interactions occur between these hemoglobins and other components, e.g., cellulose, starch, or ampholytes, in one or more of these systems. In any case, the isoelectric points of these hemoglobins are well below the pH normally used for electrophoresis of fish hemoglobins (pH 8-9) and therefore, based on electrophoretic properties alone, Hb M2A and Hb M2B would be designated as anodal hemoglobins.

It was suprising to find then, that in terms of their oxygen binding properties, Hb M2A and Hb M2B behave like cathodal hemoglobins. The oxygen equilibrium of Hb M2B is independent of pH from pH 6.2 to pH 8.0 and is unaffected by ATP (at pH 7.1). Temperature affects mainly the cooperativity of oxygen binding with the oxygen affinity essentially independent of temperature (ΔH_{av} = +0.4 kcal /mol). The oxygen binding properties of Hb M2A are essentially identical to those of Hb M2B except that the oxygen equilibrium appears to be pH dependent. The simultaneous increase in affinity and decrease in cooperativity seen at pH 6.2 and at pH 8.0 suggest that Hb M2A may be dissociating into dimers at these extremes of pH. Human hemoglobin shows a similar increased affinity and decreased cooperativity as the hemoglobin concentration is decreased below about 60 uM (139), reflecting greater dissociation to dimers as the concentration approaches the value of the tetramer-dimer dissociation constant of oxyhemoglobin ($K_{4,2} = 10^{-6}$ M). Also, it has been shown (140) that $K_{4,2}$ for human hemoglobin is pH dependent.

In the oxygen equilibrium measurements for cutthroat trout hemoglobins, the hemoglobin concentration in most cases was 20-30 uM, and since values $K_{4,2}$ for oxyhemoglobin of fishes are smaller

($K_{4,2}$ = 10^{-8} to 10^{-9} M (32)) than for human hemoglobin, dissociation to dimers should be minimal and have little or no effect on the oxygen equilibria. For Hb M2A however, only small quantities of protein were obtained and the concentration used for oxygen equilibrium measurements was 5-10 uM. At this concentration, dissociation to dimers may be expected to affect the oxygen equilibrium curve if $K_{4,2}$ is greater than 10^{-7} M. If dissociation to dimers is the cause of the pH dependence observed for the oxygen equilibrium of Hb M2A, the results suggest that $K_{4,2}$ is minimal ($< 10^{-7}$ M) at neutral pH and increases ($> 10^{-7}$ M) as the pH is raised or lowered from this value.

There is only one report in the literature describing fish hemoglobins with properties similar to those observed for Hb M2A and Hb M2B (electrophoretically anodal but with oxygen binding properties resembling cathodal hemoglobins). Iuchi (88) examined the hemoglobins of larval rainbow trout (alevins), 1 day after hatching of eggs. Starch gel electrophoresis of the larval red blood cell hemolyzate resolved 9 hemoglobins, all migrating towards the anode at pH 8.6. The mobilities of the larval hemoglobins differ from those of the 6 anodal hemoglobins present in adult hemolyzates. The visible absorption spectra of whole larval and adult hemoglobins are essentially identical, and the larval hemoglobins appear to be typical tetrameric hemoglobins by gel-exclusion chromatography and ultracentrifuge analysis. Oxygen equilibrium measurements in the pH 6.5-9.0 range show that both the affinity and cooperativity of oxygen binding for the whole larval hemoglobin are independent of pH.

Based on the properties of larval hemoglobins of rainbow trout as described by Iuchi and the observed properties of Hb M2A and Hb M2B from cutthroat trout, it is proposed that these two hemoglobins are 'larval` hemoglobins which are present in red blood cell hemolyzates from adult cutthroat trout. Electrophoresis and isoelectric focusing patterns suggest that very similar hemoglobins are present in adult rainbow trout hemolyzates. As described below, these hemoglobins may be better classified as 'embryonic` hemoglobins in accordance with the accepted definitions for hemoglobins of early development.

The changes in red blood cell type and in the hemoglobin present in these cells during development from embryo to adult appear to follow similar patterns in most vertebrates (141, 142). The first circulating erythrocytes in the embryo are nucleated and contain ribosomes, mitochondria, and other cytoplasmic organelles. This 'primitive` cell line contains hemoglobins, defined as embryonic hemoglobins, which are structurally and functionally distinct from those of adults. As the embryo develops these primitive erythrocytes are replaced by cells from the 'definitive` cell line, which constitute the circulating erythrocyte population for the remaining life span of the organism. In most mammals, two types of hemoglobins, fetal and adult, are found in these cells. The fetal hemoglobin(s) predominate in the fetus during pregnancy and are replaced by the adult hemoglobins after birth. A similar transition occurs during the development of some other vertebrates. Larval hemoglobins, analagous to the fetal hemoglobins of mammals, are expressed during the transition from embryo to adult. In other

vertebrates only embryonic and adult hemoglobins are found. Most or all of the cytoplasmic organelles, including the nucleus in mammals (but not in other vertebrates), are absent from the circulating erythrocytes derived from the definitive cell line. The switch from embryonic to fetal or adult hemoglobins and from primitive to definitive cell lines is also accompanied by a change in the site of erythopoisis (erythocyte formation). Erythropoisis initially occurs only in the yolk sac and shifts to other organs as the yolk sac disappears and these organs form in the developing embryo.

In rainbow trout, the population of circulating erythocytes of the primitive type declines within a few days after hatching of eggs and is replaced by definitive type cells (143, 144). This change in red blood cell type parallels the transition from larval to adult hemoglobins. Larval hemoglobins cannot be detected by starch gel electrophoresis in rainbow trout fry 25 days after hatching (145). It appears that the presence of these hemoglobins in adult fishes is not analagous to the presence of small amounts of fetal hemoglobin in adult human red blood cells. Rather, the larval hemoglobins of trout are analagous to the embryonic hemoglobins of other vertebrates, as they are associated with the primitive cell line of erythrocytes and appear to be the first hemoglobins synthesized in the embryo. Therefore these hemoglobins (Hb M2A and Hb M2B) are referred to as embryonic hemoglobins in the subsequent discussion.

The juvenile hemoglobins reported in young individuals of some fishes are more properly classified as larval hemoglobins. It remains to be shown however, that structurally and functionally distinct larval hemoglobins occur in teleosts. The juvenile hemo-

globins of the Atlantic salmon, Salmo salar, described by Wilkens (29) are more accurately described as a subset of the adult hemoglobins. Tsuyuki and Ronald (30) claim to find one globin chain unique to juvenile sockeye salmon, Oncorhynchus nerka, but they present little experimental evidence to support this claim. The results of Shimada et al. (28) suggest that most if not all of the juvenile hemoglobins reported in the eel (78) are actually found in high proportions in adults. No functional studies have been reported for any of these juvenile hemoglobins. These reports of juvenile or larval hemoglobins in fishes are based soley on electrophoretic patterns, and given the possibilites for artifacts, need to be substantiated by other further characterization of the putative larval hemoglobins.

A comparison of the functional properties of Hb M2A and Hb M2B with those of embryonic hemoglobins of other vertebrates lends some support to the proposal that Hb M2A and Hb M2B are embryonic hemoglobins. In general, the oxygen equilibria of embryonic hemoglobins are characterized by higher oxygen affinities and decreased sensitivities to heterotropic effectors than adult hemoglobins. For example, the value of log $p_{1/2}$ for the four embryonic hemoglobins of the chicken (at pH 7.2, 37°C) range from 0.27 to 0.53, while log $p_{1/2}$ for the adult hemoglobin is 0.87 (146). The magnitude of the Bohr effect for the two major embryonic hemoglobins is only 10-20% as large as that of the adult hemoglobin, and the decrease in the oxygen affinity in the presence of ATP is only half as large for the embryonic hemoglobins as for the adult

hemoglobin (146). Similar increased oxygen affinities and decreased Bohr effects have been observed for mouse embryonic hemoglobins (147) and one of the three embryonic hemoglobins of humans (148). These same general trends are well documented for the comparison of the oxygen binding properties of cathodal and anodal hemoglobins of fishes. In cutthroat trout, the cathodal hemoglobins (Hb F1, Hb F2, Hb F3, Hb F4, and Hb M1) and Hb M2A and Hb M2B have higher oxygen affinities (log $p_{1/2}$ = 1.28-1.34) than the anodal hemoglobins Hb S1 and Hb S2 (log $p_{1/2}$ ~ 1.5) at pH 7.1 and 20°C. The oxygen equilibria of the cathodal hemoglobins and Hb M2A and Hb M2B are all devoid of heterotropic effects due to protons and ATP, with the possible exception of a pH effect for Hb M2A.

The functional similarities between these hemoglobins of cutthroat trout and embryonic hemoglobins of higher vertebrates probably reflect similar structural features in these two groups of hemoglobins. In their determination of the amino acid sequence of the α-type globin chain of human embryonic hemoglobin, Clegg and Gagnon (149) point out the similarities between this sequence and those of the α chains from fishes. Most strikingly, the N-terminal residue of the human embryonic α chain is acetylserine (150), the same as in most and perhaps all fish hemoglobins. Based on their amino acid sequence for the human embryonic α-type chain (ζ) and on the nucleotide sequences of human globin genes, these authors conclude (149, p. 6079) that "the estimated divergence of the ζ and α genes [occurred] at ~ 400 million years ago, about the time that the line leading to bony fish became established." Therefore, the α-type chain(s) of embryonic hemoglobins of trout may have arose from

the same ancestral gene which evolved to code for this polypeptide in humans and other higher vertebrates. Given that the observed properties of cathodal hemoglobins from cutthroat trout are similar to those of the supposed embryonic hemoglobins Hb M2A and Hb M2B (and to embryonic hemoglobins in general), the α globin chains of the cathodal hemoglobins of trout may also have evolved from this ancestral α-type globin gene. If Hb M2A and Hb M2B are embryonic hemoglobins, then the amino acid sequence(s) of the α chains would provide some interesting information in regard to the molecular evolution of hemoglobin in vertebrates.

There is no direct evidence that Hb M2A and Hb M2B are in fact embryonic hemoglobins. These hemoglobins may be either larval hemoglobins or adult hemoglobins present in low proportions. The fact that embryonic hemoglobins are not normally detectable in circulating erythrocytes of adult mammals (151) might also be used as an argument against this proposal. Since embryonic hemoglobins are associated with the primitive erythrocytes derived from the yolk sac, it has been presumed that the switch from embryonic to fetal or adult hemoglobins is an irreversible process associated with the disappearance of the yolk sac and subsequent proliferation of the definitive cell line in which the genes for the embryonic globins are not expressed.

Definitive cell lines do however have the potential to express embryonic globin genes. In humans with α-thalassemia, a genetic disorder in which functional adult α chains are not produced, the embryonic α-type chain is found in circulating erythrocytes of

newborns (152), long after the yolk sac has disappeared. In this case "the expression of an embryonic-type globin gene in a definitive erythriod cell line may be the result of selective processes since the complete absence of an α-like chain would produce fetal death after about 30 days of intrauterine life." (151, p. 630) Recent results suggest that, in some vertebrates, embryonic hemoglobins are found in definitive erythroctes under less extreme conditions. In the hamster (153), erythroid cells from the liver one day after birth contain a small proportion of embryonic hemoglobins. Based on unpublished results, Chapman et al. (154) have suggested that one of the α-type embryonic globin genes of the chicken may sometimes be expressed during conditions of anemia or hypoxia. Thus, the expression of embryonic globin genes in definitive erythroid cells may not be as highly repressed as was once thought.

Since embryonic globin chains can be synthesized in erythriod cell lines present after birth, and since trout are among the most primitive of vertebrates, it may not be suprising if embryonic globin genes are expressed to some degree even in adult fishes. The possibility that the expression of these genes is subject to selective pressures may be significant in this regard. For instance, if the cathodal hemoglobins evolved from the embryonic hemoglobins as suggested above, then the primitive cathodal hemoglobin may have contained one of the embryonic globin chains. If this cathodal hemoglobin provided a selective advantage to the fish, there would be a selective pressure in favor of embryonic globin gene expression in the adult fish.

If embryonic hemoglobins are indeed present in circulating erythrocytes of adult trout as is proposed, investigation of the control of globin gene expression in these fishes could provide valuable insight into the molecular mechanism of the switch from embryonic to fetal or adult hemoglobins and of control of gene expression during development in general. In light of these considerations and those regarding the molecular evolution of hemoglobin discussed above, the possibility that Hb M2A and Hb M2B are embryonic hemoglobins is worthy of further study.

In addition to comparing the oxygen binding properties of the multiple hemoglobins of cutthroat trout, the subunit compositions of the hemoglobins were studied to determine the structural relationships (and indirectly the possible genetic relationships) among these hemoglobins. The results of this aspect of the study are only preliminary in nature. Electophoresis of the globins from the purified hemoglobins shows that all are comprised of α- and β-type chains. Hb F4 and Hb M1 are hybrid tetramers of the type $\alpha\alpha'\beta_2$ and $\alpha\alpha'\beta\beta'$, respectively. The other hemoglobins (with the possible exception of Hb M2A for which globin was not obtained) are all $\alpha_2\beta_2$ tetramers. Inclusion of a non-ionic detergent in the gels greatly enhances the separation between the two types of globin chains. The detergent is thought to bind to hydrophobic regions of the polypeptides and mask nearby charged groups or alter their pKs (155). Although the α-type chains as a group are well separated from the β-type chains, the differences in mobilities within each type of chain are not sufficient to unambiguously determine the relationships

between subunits of the different hemoglobins. For example, the respective α- and β-type chains of Hb F1, Hb F2, and Hb F3 are not readily dishinguishable.

It was thought that isoelectric focusing of the globins in the presence of urea and a non-ionic detergent might provide greater resolution of the similar α- and β-type chains. The high resolution of this system was demonstrated by Saglio et al. (156) who separated two variants of the β-type chain of human fetal hemoglobin by this technique. The two chains differ only in the conservative replacement of alanine for glycine in one position. The cutthroat trout globins however, appear to behave differently than human globins in this system. The number of bands present for each of the purified globins and the relative intensity of these bands are difficult to reconcile with the patterns obtained by electrophoresis.

The apparent complexity of the isoelectric focusing patterns of cutthroat trout globins may result from a tendency of these globins to remain in tetrameric combinations under the focusing conditions. The basis for this interpretation can be most clearly seen by considering the patterns for the two anodal hemoglobins Hb S1 and Hb S2 in Fig. 42. These globins are each resolved into three bands by IEF. Two of the bands are very faint compared to the third band. A visual comparison of the intensities of these bands suggests that the amount of protein present in the most prominent band greatly exceeds that in both of the other bands combined. Therefore it does not seem likely that the two faint bands represent two variants of the α-type chain (not resolved by electrophoresis) while the darker

band represents the β-type chain. An alternative interpretation of this pattern is that the faint bands (which are of approximately equal intensity) represent the α- and β-type chains while the darker band represents the tetrameric form of the globin.

It may seem unreasonable to suggest that the globins are associated in tetramers in the presence of 8 M urea and 2% Triton X-100. The greater strength of subunit interactions in fish hemoglobins as compared to human hemoglobin has been well documented however, and it is possible that these interactions remain strong when the heme groups are removed. Edelstein et al. (32) observed no appreciable dissociation of carp hemoglobin in the presence of urea (up to 6M) at pH 7, although denaturation was apparent at the higher urea concentrations. In contrast, human hemoglobin was completely dissociated to dimers in 2 M urea. Hb I and Hb IV of rainbow trout are not affected by other chaotropic agents (4 M KI, 0.5 M triethylamine) which cause dissociation of human hemoglobin, and globins prepared from Hb I and Hb IV retain approximately 60% of the α-helical structure present in the hemoglobins (157). Brunori and coworkers (93, 158) were able to separate the globins of Hb I and Hb IV by starch gel electophoresis as described by Chernoff and Pettit (129) (in gels containing 8 M urea, 50 mM 2-mercaptoethanol, and 0.1 M barbital, pH 8.0), however, they also refer to unusual behavior of the rainbow trout globins during ion-exchange chromatography under similar conditions (citation [8] in 93, 158) without providing any details. Several attempts to separate cutthroat trout globins by the same electrophoretic technique and similar electro-

phoretic techniques (136) were unsuccessful. If cutthroat trout globins do maintain their ability to associate in tetramers in 8 M urea and 2% Triton X-100, this finding may be valuable in understanding the nature of the subunit interactions in these hemoglobins.

Although the results of isoelectric focusing are interesting in this respect, they are not very useful in our attempt to determine the structural relationships among cutthroat trout hemoglobins. Some speculation regarding these relationships is possible based on electrophoresis and isoelectric focusing of the globins. However, given the number of uncertainties involved in any analysis of the patterns on these gels, the usefulness of such speculation is limited. Further studies of the individual globins, either by amino acid (or globin gene nucleotide) sequencing or immunological techniques, may be required to clarify the nature of these relationships. One interesting feature of the electrophoresis pattern is an apparent similarity of one of the α-type chains of Hb M2B with one of the α-type chains of Hb M1 and Hb F4, the two hemoglobins which contain two different α-type chains, and possibly with the α-type chain of Hb S2. A similarity in the globins of these four hemoglobins is also apparent by isoelectric focusing (the most anodal of the faint bands is present in all four globins). Thus, these preliminary results suggest a possible structural relationship between Hb M2B, which is proposed to be an embryonic hemoglobin, to both the anodal and the cathodal hemoglobins in cutthroat trout.

Nine hemoglobins have been isolated from blood of cutthroat trout. All are typical vertebrate hemoglobins in that they bind oxygen cooperatively and are tetramers with molecular weights of ~ 64,000, composed of α- and β-type globin chains. Six of the hemoglobins are composed of two identical α-type chains and two identical β-type chains. Two of the hemoglobins, Hb F4 and Hb M1, are hybrid tetramers composed of three and four different globin chains, respectively.

These hemoglobins have been classified in three groups; cathodal, anodal, and embryonic. The five cathodal hemoglobins, Hb F1, Hb F2, Hb F3, Hb F4, and Hb M1, have isoelectric points between 9 and 7. The oxygen equilibria of the cathodal hemoglobins are characterized by an absence of heterotropic effects due to protons and ATP, and overall enthalpies of oxygenation ~ 0. The two anodal hemoglobins, Hb S1 and Hb S2, have isoelectric points near 6.5 and are characterized by oxygen binding properties markedly different than those of the cathodal hemoglobins. both the affinity and cooperativity of oxygen binding to Hb S1 and Hb S2 are strongly pH dependent. Saturating levels of ATP or GTP cause a large decrease in the oxygen affinity for both hemoglobins, while the cooperativity of oxygen binding is essentially unaffected. In the presence of these nucleotides the Root effect for a mixture of Hb S1 and Hb S2 is shifted to higher pH. The oxygen affinity of the anodal hemoglobins is strongly temperature dependent, with both Hb S1 and Hb S2 having an overall enthalpy of oxygenation of -14 kcal/mol at pH 7.1. The remaining two hemoglobins, Hb M2A and Hb M2B, are present in low proportions in crude hemolyzates and are tentatively classified as

embryonic hemoglobins. This designation is based on a comparison of the observed electrophoretic and oxygen binding properties of these hemoglobins to those described by Iuchi (88) for the hemoglobins from newly-hatched rainbow trout. Hb M2A and Hb M2B have isoelectric points near 5.9 and oxygen binding properties essentially identical to those observed for the five cathodal hemoglobins. The results of these studies on the hemoglobins of cutthroat trout are summarized in Table X.

These results indicate that that the presence of multiple hemoglobins in cutthroat trout does not result in different oxygen transport properties for cutthroat trout blood as compared to the blood of rainbow trout. By the criteria tested, the five cathodal hemoglobins of cutthroat trout have identical oxygen binding properties, similar or identical to those previously described for the two cathodal hemoglobins of rainbow trout. Our results do suggest, however, that hemoglobin oxygen affinity may be regulated differently in these two fishes. The concentration of GTP in the red blood cells of cutthroat trout is considerably higher than in red blood cells of rainbow trout, where only ATP occurs at concentrations high enough to significantly affect the oxygen affinity of the (anodal) hemoglobins which are sensitive to nucleotides. Both ATP and GTP may be important physiological effectors of the anodal hemoglobins in the red blood cells of cutthroat trout.

The ability to regulate hemoglobin oxygen affinity by changes in red blood cell ATP and/or GTP concentrations may allow a greater

TABLE X

Summary of properties of cutthroat trout hemoglobins

Hemoglobin	Isoelectric point[a]	Subunit type[b]	Oxygen equilibrium: Affected by H+?	Affected by ATP?	ΔH_{av}[c] (kcal/mol)
Cathodal:					
F1	9.0	$\alpha_2\beta_2$	no	no	+0.4
F2	8.5	$\alpha_2\beta_2$	no	no	+0.2
F3	8.1	$\alpha_2\beta_2$	no	no	+0.6
F4	7.8	$\alpha\alpha'\beta_2$	no	no	+0.6
M1	7.1	$\alpha\alpha'\beta\beta'$	no	no	+0.4
Embryonic:					
M2A	<5.9	?	?	no	+0.8
M2B	5.9	$\alpha_2\beta_2$	no	no	+0.4
Anodal:					
S1	6.6	$\alpha_2\beta_2$	yes	yes	-14
S2	6.5	$\alpha_2\beta_2$	yes	yes	-14

[a] Determined by isoelectric focusing at 24 +/- 1.5°C.

[b] No identities of subunits for the different hemoglobins are implied.

[c] Overall enthalpy of oxygenation, obtained from the van't Hoff plot of log $p_{1/2}$ (at pH 7.1) from 10°C to 30°C and corrected for the heat of solution of oxygen (-3.1 kcal/mol at 20°C).

flexibility in the control of red blood cell metabolism and oxygen transport. Further studies of red blood cell nucleotide concentrations in rainbow and cutthroat trout under varying physiological conditions, e.g., exercise, hypoxia, temperature stress, should be performed to investigate this possible difference between rainbow and cutthroat trout.

Two other areas deserving further study are the structural relationships between the multiple hemoglobins of cutthroat trout and the nature of changes in globin gene expression during development in trout. Information obtained from these studies could provide an understanding of the genetic basis for the high degree of hemoglobin multiplicity in cutthroat trout and other fishes. These studies may also yield valuable insight regarding the evolution of globin genes in vertebrates and the regulation of expression of these genes during development.

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APPENDIX

Appendix A. Computer program for calculation of hemoglobin concentrations

```
100 REM   PROGRAM   "HBCONC"
200 REM
300 REM   VERSION 5        JAN. 7, 1982
400 REM   PROGRAMMER\      JON SOUTHARD
500 REM
600 REM   NORTHSTAR BASIC TRANSLATION
700 REM   OF "HBCALC (FORTRAN)"
800 REM
900 REM   AUTHOR\ TED MIFFLIN      MARCH 21, 1981
1000 REM
1100 REM
1200 REM    CALCULATES HEMOGLOBIN CONCENTRATIONS
1300 REM    FROM A560, A576, A630
1400 REM
1500 REM    READS ABSORBANCE DATA DIRECTLY
1600 REM    OR FROM DATA FILE "SPECDAT"
1700 REM
1800 REM    READS PH DEPENDENT EXTINCTION COEFFICIENTS
1900 REM    FROM DATA FILE "EXCODAT"
2000 REM
2100 REM    PRINTS ABSORBANCE DATA AND WRITES
2200 REM    CONCENTRATIONS TO DATA FILE "CONCDAT"
2300 REM
2400 REM    ******************************************
2500 REM    INPUT/MATRIX FORMATION SECTION
2600 REM
2700 DIM A(3,4),B(3,4),C(4,30),I1(3),L1(3),X(3),C1(3),P(3)
2800 N=3\ N1=N+1\ R=15\Z3=0
2900 REM
3000 REM    READ OXY AND DEOXY COEFFICIENTS
3100 REM
3200 FOR I=1 TO N\ FOR J=1 TO N-1
3300 READ A(I,J)
3400 DATA 0.906,1.340,1.650,1.010,0.0150,0.115
3500 NEXTJ\ NEXT I
3600 REM
3700 REM    INPUT # OF DATA SETS AND PH
3800 REM
3900 Z1=1\Z2=2
4000 INPUT "NUMBER OF DATA SETS= ",Y1
4100 INPUT "PH= ",P1
4200 REM
4300 REM    READ MET COEFFICIENTS
4400 REM
4500 OPEN #5,"EXCODAT"
4600 FOR I=1 TO N1\ FOR L=1 TO 30
4700 READ #5,C(I,L)
4800 NEXT L\ NEXT I
4900 CLOSE #5
5000 FOR L=1 TO 30
5100 IF C(4,L)=P1 THEN 5300
5200 NEXT L
```

```
5300 L2=L
5400 FOR I=1 TO 3
5500 A(I,3)=C(I,L2)
5600 NEXT I
5700 REM
5800 REM    INPUT/READ ABSORBANCE DATA
5900 REM
6000 OPEN #4,"SPECDAT"
6100 INPUT "IS ABSORBANCE DATA STORED? ",Q$
6200 IF Q$="NO" THEN 6400
6300 Z1=Z2 \ GOTO 7100
6400 !#Z1,"ENTER A560, A576, A630"
6500 FOR I=1 TO Y1
6600 !#Z1,"N= ",%3I,I
6700 INPUT "> ",A(1,4),A(2,4),A(3,4)
6800 WRITE #4%(I-1)*R,A(1,4),A(2,4),A(3,4),NOENDMARK
6900 IF Z3=1 THEN 8500
7000 NEXT I
7100  !#Z1,"  N  A560  A576  A630    PH ",%3F1,P1
7200 FOR I=1 TO Y1
7300 READ #4%(I-1)*R,A(1,4),A(2,4),A(3,4)
7400 REM
7500 REM    PRINT ABSORBANCE DATA
7600 REM
7700  !#Z1,%3I,I,%6F4,A(1,4),A(2,4),A(3,4)
7800 NEXT I
7900 IF Q$="YES" THEN 9300
8000 IF Z1=Z2 THEN 9300
8100  INPUT "HOW MANY DATA SETS NEED CORRECTION? ",Q
8200 Z3=1 \ IF Q=0 THEN 8600
8300  FOR V=1 TO Q \ INPUT "N FOR BAD DATA= ",Z
8400  I=Z \ GOTO 6600
8500 NEXT V
8600 Z1=Z2 \ GOTO 7100
8700 REM
8800 REM    ***********************************
8900 REM          SUBROUTINE GAUJOR
9000 REM
9100 REM    SOLVES THE 3X4 MATRIX
9200 REM
9300 OPEN #3,"CONCDAT"
9400 FOR S=1 TO Y1
9500 READ #4%(S-1)*R,A(1,4),A(2,4),A(3,4)
9600 GOSUB 9900
9700 IF I2=1 THEN 15200
9800 GOTO 1070
9900 N1=N+1 \ I2=0
10000 FOR I=1 TO N \ FOR J=1 TO N1
10100 B(I,J)=A(I,J)
10200 NEXT J \ NEXT I
10300 FOR I=1 TO N \I1(I)=0 \ NEXT I
10400 REM
```

```
10500 REM   MAIN LOOP, ELIMINATE ONE COLUMN AT A TIME
10600 REM
10700 FOR I=1 TO N \ I3=I+1
10800 REM
10900 REM   FIND MAXIMUM ELEMENT IN THE ITH COLUMN
11000 REM
11100 A1=0
11200 FOR K=1 TO N
11300 E=ABS(B(K,I))
11400 IF E<A1 THEN 12000
11500 REM
11600 REM   HAS THIS ROW BEEN USED AS A PIVOT ROW?
11700 REM
11800 IF I1(K)=1 THEN 12000
11900 L1(I)=K \ A1=E
12000 NEXT K
12100 REM
12200 REM   IS THE COEFFICIENT MATRIX SINGULAR?
12300 REM
12400 IF A1<1.0E-35 THEN 14200
12500 L=L1(I)\I1(L)=1
12600 FOR J=1 TO N
12700 IF J=L THEN 13200
12800 F=-B(J,I)/B(L,I)
12900 FOR K=I3 TO N1
13000 B(J,K)=B(J,K)+F*B(L,K)
13100 NEXT K
13200 NEXT J
13300 NEXT I
13400 REM
13500 REM   CALCULATE THE SOLUTION VECTOR, X
13600 REM
13700 FOR I=1 TO N
13800 L=L1(I)
13900 X(I)=B(L,N1)/B(L,I)
14000 NEXT I
14100 RETURN
14200 I2=1
14300 RETURN
14400 REM   ************************************
14500 REM         OUTPUT SECTION
14600 REM
14700 REM   WRITE CONCENTRATIONS TO DATA FILE
14800 REM
14900 WRITE #3%(S-1)*R,C1(1),C1(2),C1(3),NOENDMARK
15000 NEXT S
15100 GOTO 15300
15200 !#Z1,"PROGRAM FAILURE DUE TO PIVOT ELEMENT <1.0E-35"
15300 CLOSE #4 \CLOSE #3
15400 END
```

```
100 REM    PROGRAM    "DATFIL"
200 REM
300 REM    JON SOUTHARD    DEC. 13, 1981
400 REM
500 REM    CREATES DATA FILE "EXCODAT"
600 REM    FOR PROGRAM "HBCONC"
700 REM
800 REM*****************************************************
900  REM          MET Hb EXTINCTION COEFFICIENTS
1000 REM
1100 REM    AT 560nm
1200 DATA .357,.357,.358,.358,.361,.363,.368,.372,.380,.387
1300 DATA .397,.406,.418,.430,.445,.460,.479,.497,.520,.542
1400 DATA .566,.589,.613,.636,.658,.679,.698,.716,.735,.753
1500 REM
1600 REM    AT 576nm
1700 DATA .341,.342,.343,.344,.349,.354,.361,.368,.378,.388
1800 DATA .401,.414,.430,.445,.465,.485,.510,.534,.564,.593
1900 DATA .623,.652,.683,.713,.739,.765,.789,.813,.837,.861
2000 REM
2100 REM    AT 630nm
2200 DATA .395,.394,.393,.392,.391,.391,.390,.388,.386,.384
2300 DATA .380,.376,.370,.363,.354,.344,.327,.310,.302,.293
2400 DATA .281,.268,.256,.243,.232,.220,.220,.199,.199,.198
2500 REM
2600 REM    FOR  THE pH VALUES
2700 DATA 6.1,6.2,6.3,6.4,6.5,6.6,6.7,6.8,6.9,7.0
2800 DATA 7.1,7.2,7.3,7.4,7.5,7.6,7.7,7.8,7.9,8.0
2900 DATA 8.1,8.2,8.3,8.4,8.5,8.6,8.7,8.8,8.9,9.0
3000 REM*****************************************************
3100 OPEN #5,"COEXDAT"
3200 DIM A(120)
3300 R=5
3400 FOR X=1 TO 120
3500 READ A(X)
3600 WRITE #5%(X-1)*R,A(X)
3700 NEXT X
3800 !#1,"ALL DATA ENTERED"
3900 CLOSE #5
4000 END
```

Appendix B. Oxygen equilibrium parameters of cutthroat trout hemoglobins

TABLE XI

Oxygen equilibrium parameters of Hb F1, Hb F2, and Hb F3

Values of $n_{1/2}$ and log $p_{1/2}$ from the oxygen equilibrium curves. Measurements at pH 7.1 and 20°C, except as indicated. Numbers in parentheses are the number of determinations (where not indicated, only one determination was made). See Materials and Methods for details.

Variable	Hb F1		Hb F2		Hb F3	
	$n_{1/2}$	log $p_{1/2}$	$n_{1/2}$	log $p_{1/2}$	$n_{1/2}$	log $p_{1/2}$
pH:						
8.0	2.3	1.22	2.2	1.26	2.2	1.29
7.4	2.3	1.29	2.2	1.34	2.2	1.34
7.1	2.3	1.31(3)	2.3	1.33(3)	2.2	1.34(2)
6.8	2.2	1.23			2.3	1.28
6.2	2.0	1.24	2.3	1.31	2.1	1.28
Nucleotides:						
ATP	2.3	1.32	2.3	1.33	2.2	1.34
Temperature:						
30°C	1.8	1.39	1.6	1.44	1.7	1.42
10°C	3.0	1.25	2.5	1.29	2.6	1.29

TABLE XII

Oxygen equilibrium parameters of Hb F4, Hb M1, and Hb M2A

Values of $n_{1/2}$ and log $p_{1/2}$ from the oxygen equilibrium curves. Measurements at pH 7.1 and 20°C, except as indicated. Numbers in parentheses are the number of determinations (where not indicated, only one determination was made). See Materials and Methods for details.

	Hb F4		Hb M1		Hb M2A	
Variable	$n_{1/2}$	log $p_{1/2}$	$n_{1/2}$	log $p_{1/2}$	$n_{1/2}$	log $p_{1/2}$
pH:						
8.0	2.3	1.30	2.1	1.26	1.7	0.90
7.4	2.4	1.33	2.2	1.35		
7.1	2.2	1.34(2)	2.2	1.34(2)	2.0	1.28
6.8	2.4	1.27	2.3	1.28		
6.2	2.3	1.32	2.2	1.30	1.7	0.86
Nucleotides:						
ATP	2.3	1.34	2.2	1.34	2.0	1.27
Temperature:						
30°C	1.8	1.40	1.7	1.42		
10°C	3.0	1.27	2.6	1.28	2.3	1.22

TABLE XIII

Oxygen equilibrium parameters of Hb M2B, Hb S1, and Hb S2

Values of $n_{1/2}$ and log $p_{1/2}$ from the oxygen equilibrium curves. Measurements at pH 7.1 and 20°C, except as indicated. Numbers in parentheses are the number of determinations (where not indicated, only one determination was made). See Materials and Methods for details.

	Hb M2B		Hb S1		Hb S2	
Variable	$n_{1/2}$	log $p_{1/2}$	$n_{1/2}$	log $p_{1/2}$	$n_{1/2}$	log $p_{1/2}$
pH:						
8.0	2.0	1.25(3)	1.9	1.06	1.9	1.04
7.8			1.7	1.15	1.9	1.12
7.4	2.1	1.35	2.1	1.26	2.0	1.26
7.1	2.0	1.31(3)	2.3	1.52	2.2	1.50
6.8			2.3	1.90	2.0	1.89
6.5			2.0	2.38	2.0	2.35
6.2	2.1	1.27(2)	1.2[a]	2.7[a]	0.9[a]	2.8[a]
Nucleotides:						
ATP	2.0	1.30(2)	2.5	2.07	2.1	2.07
GTP			2.5	2.12	2.3	2.12
Temperature:						
30°C	1.6	1.43	2.0	1.95	1.8	1.94
25°C			2.2	1.77		
15°C			2.4	1.32	2.1	1.27
10°C	2.5	1.29	2.4	1.09	2.2	1.06

[a]Estimated values, see Fig. 30.

VITA

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